

Evaluation of GSH adducts of adrenaline in biological samples

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ABSTRACT: The sustained high release of catecholamines to circulation is a deleterious condition that may induce toxicity, which seems to be partially related to the products formed by oxidation of catecholamines that can be further conjugated with glutathione (GSH). The aim of the present study was to develop a method for the determination of GSH adducts of adrenaline in biological samples. Two position isomers of the glutathion-S-yl-adrenaline were synthesized and characterized by HPLC using diode array, coulometric and mass detectors. A method for the extraction of these adducts from human plasma was also developed, based on adsorption to activated alumina, which showed adequate recoveries and proved to be crucial in removing interferences from plasma. The selectivity, precision and linearity of the method were all within the accepted values for these parameters. Furthermore, the sensitivity of this method allows the detection of adduct amounts that are within the range of the expected concentrations for these adducts under certain pathophysiological conditions and/or drug treatments. In conclusion, the development of this method allows the direct analysis of GSH adducts of adrenaline in human plasma, providing a valuable tool for the study of the catecholamine oxidation process and its related toxicity. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: GSH adducts; adrenaline; catecholamines; oxidative stress; HPLC

INTRODUCTION

The maintenance of high extracellular and circulating levels of catecholamines is a deleterious condition that may induce cardiotoxicity (Dhalla *et al.*, 2001; Remião *et al.*, 2001, 2002, 2004) and neurotoxicity (Spencer *et al.*, 1998; Smythies *et al.*, 2002). Excessive release of catecholamines into the interstitial space and circulation may result from stress due to Western lifestyles (Dhalla *et al.*, 1996), reduced physical activity and hypercaloric nutrition (Rupp *et al.*, 1994), or pathological conditions such as ischemia (Akiyama and Yamazaki, 2001) and pheochromocytoma (Hoffman, 2001). Also, psychoactive designer drugs of abuse such as *d*-amphetamine

and 3,4-methylenedioxymethamphetamine (MDMA, 'ecstasy') are known to induce an acute massive release of catecholamines (Hoffman and Lefkowitz, 1996). MDMA in particular can be metabolically bioactivated to highly reactive catechol metabolites, which have shown neurotoxic, hepatotoxic, cardiotoxic and nephrotoxic effects in several *in vitro* studies (Carvalho *et al.*, 2002, 2004a–c; Capela *et al.*, 2006; Carmo *et al.*, 2006). The neurotoxic effects induced by the oxidation of dopamine in the brain have also been reported to be involved in Parkinson's disease (Spencer *et al.*, 1998; Smythies *et al.*, 2002).

The underlying mechanism of toxicity mediated by catecholamines is partially due to their oxidation process. Catecholamines can be two-electron oxidized into *ortho*-quinones, through the intermediate *ortho*-semiquinones and reduction of O₂ to superoxide radical (O₂^{•-}; Fig. 1). This oxidation can occur by autoxidation, which is a slow process at physiological pH (Bindoli *et al.*, 1992; Remião *et al.*, 2001). However, this process can be accelerated under physiological conditions by enzymatic or non-enzymatic catalysis (Remião *et al.*, 2003). In fact, catecholamines are oxidized by enzymes such as xanthine oxidase (Foppoli *et al.*, 1997), peroxidase (Bindoli *et al.*, 1992) or tyrosinase (Heacock, 1959),

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Abbreviations used: GSH, glutathione; MDMA, 3,4-methylenedioxymethamphetamine.

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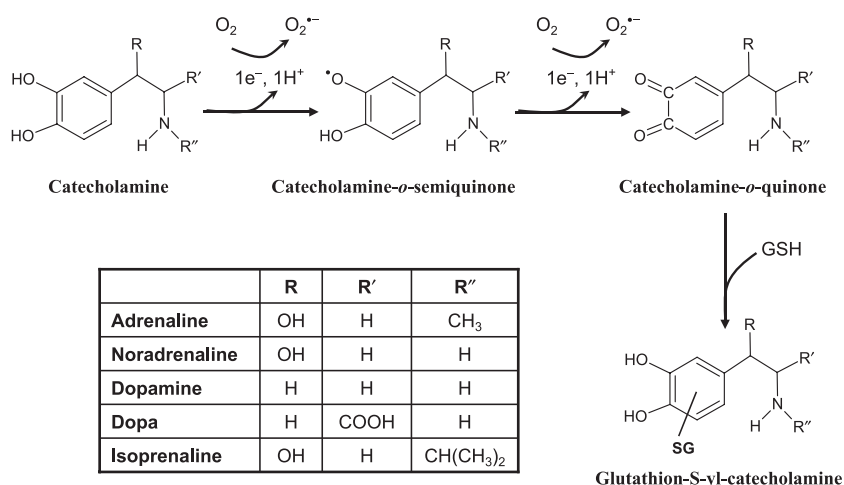


Figure 1. Proposed mechanism for glutathion-S-yl-catecholamine formation through the catecholamine oxidation process.

and in the presence of metal cations, such as Cu²⁺, Mn²⁺, Co²⁺, Ni²⁺, Fe³⁺, and several Cu²⁺ and Fe³⁺ chelates (Bindoli *et al.*, 1992). *Ortho*-quinones can undergo an irreversible 1,4-intramolecular cyclization with the formation of unstable leucoaminochromes, which are subsequently oxidized to aminochromes, through the intermediate leucoaminochrome-*ortho*-semiquinone (Bindoli *et al.*, 1989; Remião *et al.*, 2004). *Ortho*-quinones can also be conjugated with nucleophiles, especially thiols, like cysteine and glutathione, or even with protein sulfhydryl groups (Fig. 1; Bindoli *et al.*, 1992; Monks and Lau, 1998; Bolton *et al.*, 2000). This nucleophilic addition to a quinone leads to its reduction to the catechol and can occur spontaneously or catalyzed by glutathion-S-transferase (Dagnino-Subiabre *et al.*, 2000) or tyrosinase (Spencer *et al.*, 1998). The ability of the quinones to form multiple nucleophilic addition products and the ability of the quinol-thioethers to redox-cycle and generate reactive oxygen species provides a clear basis for their biological reactivity (Monks and Lau, 1997). The polyphenolic-GSH conjugates have already been shown to contribute to the nephrotoxicity, nephrocarcinogenicity and neurotoxicity of a variety of polyphenols (Monks and Lau, 1998; Monks *et al.*, 2004).

Adrenaline is secreted by the adrenal medulla and plays a key role in the short-term stress reaction. When released into the bloodstream, adrenaline binds to multiple receptors and has numerous effects throughout the body. Plasma levels of adrenaline in healthy volunteers at rest are similar to plasma levels of dopamine but lower than plasma levels of noradrenaline, which normally average around 1 nM (Goldstein *et al.*, 2003). However, plasma adrenaline concentrations increase markedly and to a greater extent than do noradrenaline concentrations in response to hypoglycemia, hemorrhagic hypotension, asphyxiation, circulatory collapse

and distress, which presumably reflects a relatively greater adrenomedullary hormonal activation than sympathetic neuronal activation (Goldstein *et al.*, 2003). Under such conditions, oxidation of the catecholamines and subsequent adduct formation is likely to occur, although no adducts between GSH and adrenaline have been described in biological samples so far. However, the presence of adducts between cysteine or GSH with dopamine has been reported in plasma of rats treated with endotoxin and dopamine (Magnay *et al.*, 2001), in the striatum of rats treated with dopamine (Byington, 1998), in amyotrophic lateral sclerosis and Parkinson's disease patients serum (Salauze *et al.*, 2005) and in substantia nigra and putamen in post-mortem brain samples of Parkinson's disease patients (Spencer *et al.*, 1998). Similarly, the glutathion-S-yl-*p*-hydroxyamphetamine conjugate has been identified *in vitro* after incubation of freshly isolated hepatocytes with *d*-amphetamine (Carvalho *et al.*, 1996). Incubation of methylenedioxyamphetamine with human liver microsomes produced both the 5- and 6-(glutathion-S-yl)- α -methyldopamine adducts and the former was shown to be behaviorally active (Easton *et al.*, 2003). The formation of the GSH adduct of N-methyl- α -methyldopamine by rat liver microsomes has also been documented (Hiramatsu *et al.*, 1990) and more recently the GSH and N-acetylcysteine conjugates of N-methyl- α -methyldopamine were identified *in vivo* in the brain of rats administered MDMA by subcutaneous injection (Jones *et al.*, 2005). It is currently acknowledged that these thioether metabolites of α -methyldopamine and N-methyl- α -methyldopamine contribute to the neurotoxic effects of their parent compound MDMA (Monks *et al.*, 2004; Jones *et al.*, 2005; Capela *et al.*, 2006).

Given these recent findings on the toxicological importance of the thioether adducts of the catecholamines,

the aim of this study was to develop a method for the evaluation of the GSH adducts of adrenaline in biological samples, which could provide a valuable tool to study the contribution of the catecholamine oxidation process to the toxicity due to sustained high levels of catecholamines.

MATERIALS AND METHODS

Synthesis of adrenaline-glutathione adducts. A reaction mixture (100 μ L) of 2.5 pmol to 375 nmol adrenaline hydrochloride (Sigma-Aldrich), 0.04 IU tyrosinase (Sigma-Aldrich) and 375 nmol GSH (Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.4; Merck) was mixed in vortex for 2 min at room temperature. After 10 min resting, 400 μ L of 0.1 M phosphate buffer (pH 7.4) or human plasma were added to the reaction mixture, which was then acidified with 250 μ L of 15% perchloric acid (Merck). Following vigorous shaking, the sample was centrifuged for 5 min at 16,000 *g*. The obtained supernatant was subsequently used for the analysis of adrenaline-GSH adducts (in 5% perchloric acid).

Analysis of the adrenaline-GSH adducts by HPLC with photodiode array, mass spectrometry and coulochemical detection. HPLC analysis was performed using a Waters 2690 separations module (Waters, Milford, MA, USA), and a commercially pre-packed reverse phase cartridge of 250 mm \times 4.6 mm, Waters Spherisorb RP-18 (5 μ m) ODS2 column. The mobile phase (10% methanol in 50 mM citric acid, 0.46 mM octanesulfonic acid, adjusted to pH 3.0) was filtered through a 0.45 μ m membrane (Millipore, Madrid, Spain) and degassed under a helium stream. An isocratic elution was performed at a flow rate of 1.0 mL/min, at room temperature. Sample aliquots of 20 μ L were usually injected. A Compaq computer fitted with Millennium³² software (Waters) processed the chromatographic and spectral data.

For the spectroscopic and coulochemical analysis, a photodiode array detector (Waters model 996) and/or a Coulochem II (ESA, Chelmsford, USA) equipped with a guard cell (ESA 5020) and analytical cell (ESA 5011A) electrochemical detector were used. For some of the studies, both detectors were used, with the analytical cell of the coulochemical detector placed after the photodiode array detector. The electrochemical potential settings of the Coulochem II detector were: guard cell, +0.5 V; detector 1, -0.075 V and detector 2 (analytical detector), +0.45 V. A current of 5 μ A full-scale was used. For the voltammogram study, the detector 2 was set at 0, +0.1, +0.2, +0.3, +0.35, +0.4, +0.45 and +0.5 V with the guard cell at +0.55 V for this last potential.

For mass spectrometry analysis, an isocratic elution of 3% methanol in 10 mM aqueous ammonium acetate (pH 2.5) mobile phase was used at a flow rate of 1.0 mL/min and at room temperature. Electrospray and tandem mass spectra were acquired with a Q-TOF 2 (Micromass, Manchester). The TOF resolution was set at 9500 (50% peak valley). The capillary needle voltage was 3000 V and the source temperature was maintained at 150°C. Nitrogen was used as the nebulizer gas and argon was used as the collision gas. Cone voltage was at 30 V for MS and MS/MS and at 55–65 V for 'pseudo' MS/MS/MS (adjusted in each case for optimal signal). Collisional

induced dissociation spectra (MS/MS) were acquired by selecting the desired ion with the quadrupole section of the mass spectrometer, and colliding it in the collision cell with argon gas (measured pressure on the Penning gauge $\sim 6 \times 10^{-5}$ mbar) using a collision energy of 20–25 eV. The TOF analyzer identified the resulting product ions. Pseudo-MS/MS/MS experiments were performed by increasing the cone voltage up to 65 V, promoting fragmentation in the source, and performing an MS/MS experiment on the in-source generated fragment ions. Data acquisition was carried out with a Micromass MassLynx 3.4 data system. Sample introduction was carried out with a syringe pump at a flow rate of 10 μ L/min.

Adrenaline-glutathione adducts extraction by alumina adsorption. After synthesis, supernatant aliquots of 750 μ L, containing GSH adducts of adrenaline obtained as previously described, were added to clean tubes containing 50 mg of washed and activated alumina and pH was adjusted to values between 8.3 and 8.6 with 1.5 M Tris-15 mM EDTA buffer (pH 8.6). The tubes were vigorously shaken during 15 min in a reciprocal agitator at room temperature. Subsequently, the supernatant was removed with vacuum and the remaining alumina was washed twice with 1 mL ice-cold water. The alumina was then suspended in 0.5 mL of ice-cold water and this last fraction was transferred into Costar Spin-X 8169 microtubes packed with a 0.2 μ m nylon filtering membrane and centrifuged at 2,000 *g* for 2 min at 4°C. The filter with the alumina was transferred into a new eppendorf and the adducts were extracted from alumina with 5% perchloric acid. After 5 min resting the microtubes were mixed in vortex and centrifuged at 2,000 *g* for 2 min at 4°C. The filtered 5% perchloric acid solution contained the GSH adducts of adrenaline and 20 μ L aliquots were then injected into the HPLC system.

The recovery of the adrenaline-GSH adducts from human plasma after alumina extraction was determined in five different plasma samples. Each sample was prepared using adrenaline-GSH adducts synthesized with 0, 0.6, 3 and 15 nmol of adrenaline in the reaction mixture as previously described and subsequently divided into two aliquots. One was kept, and the other was extracted with alumina. Twenty microliters of each aliquot were then injected into the HPLC system and the peak areas of the adrenaline-GSH adducts in both chromatograms of alumina extracted and non-alumina extracted samples were compared for recovery evaluation.

Validation of the analytical procedure. For the validation of the analytical procedure several parameters including, selectivity, detection limit, linearity and precision were studied in buffer and human plasma samples spiked with known amounts of the adrenaline-GSH adducts.

Selectivity of the adrenaline-GSH adducts peaks was evaluated by two different methods:

- (1) Treatment with γ -glutamyl transpeptidase—a reaction mixture with 15 nmol of adrenaline and 375 nmol GSH was prepared in phosphate buffer as described above. Subsequently, 20 μ L of the supernatant with the GSH adducts were added to 180 μ L of phosphate buffer (0.5 M, pH 7.4) containing 1 mM CaCl₂ (Merck) with and without γ -glutamyl transpeptidase (γ -GT; EC 2.3.2.2; 8.2 IU). After vigorous shaking and 10 min resting, 100 μ L of

15% perchloric acid were added to both solutions, which were centrifuged for 5 min at 16,000 *g*. The obtained supernatants were then injected into the HPLC system.

- (2) Direct chromatographic analysis—two aliquots of the same human plasma sample were prepared as described before with two reaction mixtures for adrenaline–GSH adducts synthesis, one containing 0.6 nmol of adrenaline and the other without adrenaline. Both samples were extracted with alumina before injection into HPLC. This procedure was applied to five different human plasma samples.

The detection limit of the adrenaline–GSH adducts was determined by analysis of chromatograms after 20 μ L injection of samples of human plasma with and without alumina extraction of the adducts. These samples were prepared by spiking human plasma samples with reaction mixtures of adrenaline–GSH adducts obtained with decreasing adrenaline amounts. The detection limit for each GSH adduct was set at the lower amount of adrenaline used in the adduct synthesis for which the corresponding adduct peak area was three times greater than the background noise.

The linearity of the method was tested in buffer and in human plasma samples with and without alumina extraction of the adrenaline–GSH adducts. These samples were spiked with reaction mixtures containing 0–125 nmol adrenaline.

The precision of this analytical procedure was tested always by the same operator. Phosphate buffer or human plasma samples were spiked with adrenaline–GSH adducts synthesized with 3 nmol of adrenaline in the reaction mixture. Each sample was injected into the HPLC system with and without previous alumina extraction of the adrenaline–GSH adducts. The precision was characterized by the injection repeatability, the procedure repeatability, and the intermediate precision parameters:

- (1) The injection repeatability parameter was evaluated by injecting 20 times the same sample for each tested condition.
- (2) The procedure repeatability parameter was evaluated by injecting 20 different samples for each tested condition.
- (3) The intermediate precision parameter was evaluated by injecting repeatedly into the HPLC, on 5 consecutive days, the same sample (kept at room temperature) for each tested condition.

Evaluation of adrenaline–glutathione adducts stability and their spontaneous formation. A phosphate buffer sample was spiked with a reaction mixture for adrenaline–GSH adduct synthesis obtained with 3 nmol of adrenaline.

The stability of the synthesized adrenaline–GSH adducts in 5% perchloric acid at room temperature was evaluated by the analysis of chromatograms obtained after five injections of the same sample during a period of 8 days.

The possibility of spontaneous formation of the adrenaline–GSH adducts in 5% perchloric acid and in 1.5 M Tris–15 mM EDTA buffer (pH 8.3–8.6) was tested in two similar experiments:

- (1) A 5% perchloric acid solution containing 4 μ M adrenaline and 0.5 mM GSH was incubated at room temperature or at -80°C for 24 h with and without ascorbic acid (1 and 5 mM, final concentration).
- (2) The Tris–EDTA buffer with 4 μ M adrenaline and 0.5 mM GSH was incubated at room temperature for 15 min with or without ascorbic acid (1 and 5 mM, final concentration).

RESULTS AND DISCUSSION

Synthesis of adrenaline–glutathione adducts

Adducts between adrenaline and GSH are not commercially available. To obtain these adducts it was necessary to synthesize them using tyrosinase as the catalytic enzyme. The used procedure was based on previous works with adducts of GSH or cysteine with dopamine (Spencer *et al.*, 1998; Magnay *et al.*, 2001) or with α -methyldopamine (Carvalho *et al.*, 2004a). The time established for the catalysis reaction by tyrosinase was set based on confirmation of the complete disappearance of the adrenaline peak monitored at 279 nm after the synthesis of GSH adducts for all the tested concentrations of this catecholamine. Thus, the complete oxidation of adrenaline with the respective formation of GSH adducts was always guaranteed, and the values of adducts always refer to the amount of adrenaline used in the synthesis.

Chromatographic settings and characterization of the GSH adduct peaks

The mobile phase used in the present study was adapted from a previous work for the detection of α -methyldopamine–GSH adducts by HPLC with coulometric detection (Carvalho *et al.*, 2004a). The percentage of methanol was adjusted in order to maximize resolution and definitions of the GSH adduct peaks. Under the conditions of this study, the best methanol percentage in the mobile phase was of 10%; however, it can vary according to room temperature or to the performance of the column at the moment of the analysis.

Figure 2 shows the coulometric chromatograms of the GSH adducts formed with 15 nmol adrenaline in the reaction mixture, with and without subsequent γ -GT treatment. Two peaks can be observed that completely disappear after treatment with γ -GT, an enzyme known to cleave the γ -glutamyl bond of GSH. This result confirmed that these two peaks correspond to GSH adducts. The analysis of the UV spectra of both GSH adduct peaks showed three λ_{max} , at 232, 256 and 290 nm, which are characteristic of S-glutathionyl conjugates of catecholamines (Fig. 2; Spencer *et al.*, 1998; Carvalho *et al.*, 2004a).

Further characterization of the GSH adduct peaks was performed by HPLC-ESI-MS and by HPLC-ESI-MS/MS. Using HPLC-ESI-MS two peaks, in agreement with the HPLC-UV data, were assigned as monogluthathione conjugates at m/z $[M + 1]$ of 489 corresponding to glutathione-S-yl-adrenaline adducts. Under the same CID conditions, both glutathione conjugates show a characteristic fragmentation pattern (Fig. 3). In both cases the CID spectra of these adducts display

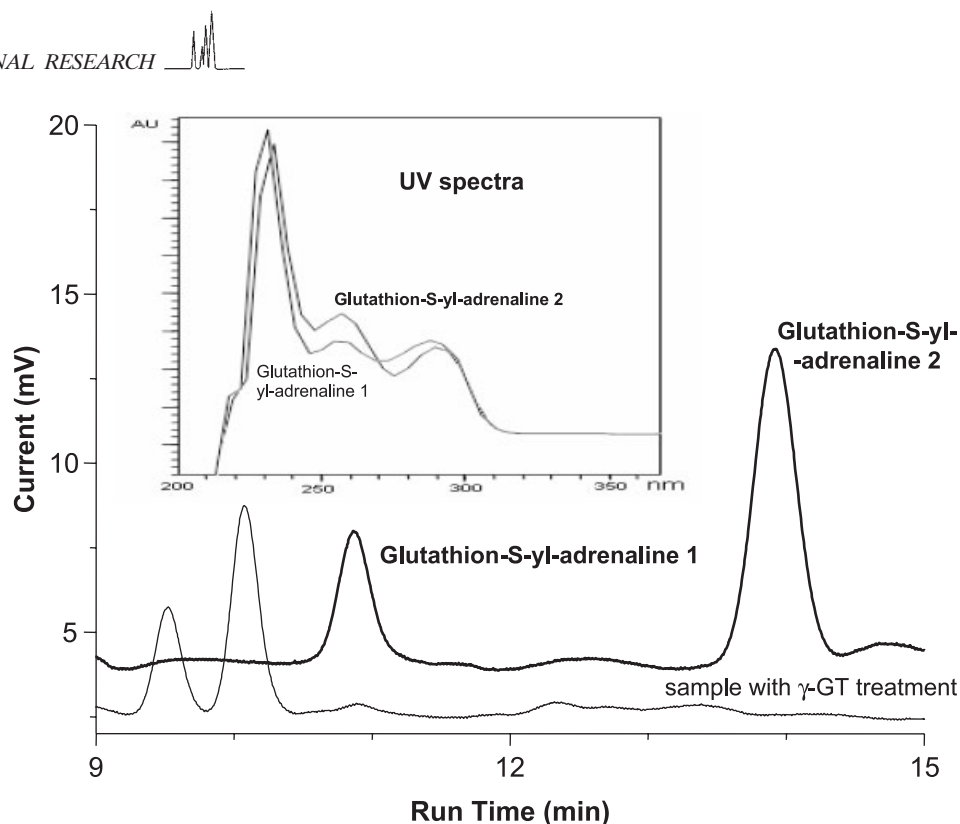


Figure 2. Coulometric chromatograms of a phosphate buffer sample spiked with a reaction mixture prepared with 15 nmol adrenaline, with and without γ -GT treatment, showing the peaks corresponding to the GSH adducts. The UV spectra of both adducts are also shown.

characteristic fragment ions of GSH conjugates derived from neutral losses of pyroglutamate residue (129 Da; Baillie and Davis, 1993) corresponding to the fragment ion at m/z 360. Other characteristic fragment ions appear at m/z 342 [M – glutamine], which in the case of glutathione-S-yl-adrenaline 2 corresponds to the spectrum base peak. In the case of glutathione-S-yl-adrenaline 1, the spectrum base peak corresponds to the fragment ion at m/z 311, derived from the loss of glutamine residue and metilamine from the adrenaline moiety [M – glutamine – NH_2CH_3]. Only in the case of compound 1, in the CID spectra, do ions appear corresponding to the glutathione moiety with the loss of the thiol group such as the ion at m/z 274 [GSH – SH], at m/z 256 [GSH – SH – H_2O] and at m/z 199 [GSH – SH – H_2O – glycine]. From the analysis and comparison of the CID spectra from the collected fractions, it is possible to state that they correspond to two different glutathione conjugated species. These results have characterized both adducts as two position isomers of glutathion-S-yl-adrenaline.

The voltammogram for both adducts was performed and is presented in Fig. 4. A response was observed for potentials higher than +0.2 V for glutathion-S-

yl-adrenaline 1 and higher than +0.0 V for glutathion-S-yl-adrenaline 2. Adduct 2 reached a plateau around +0.45 V. The settings of the detector were adapted in order to maximize the response for both adducts. The guard cell placed between the pump and the injector was set at +0.05 V higher than detector 2 to oxidize the mobile phase, thereby decreasing the background current measured by the analytical cell.

Adrenaline–glutathione adducts extraction by alumina adsorption

Alumina has long been used for plasma extraction of catecholamines (Wu and Gornet, 1985; Eisenhofer *et al.*, 1986; Carvalho *et al.*, 1997; Spencer *et al.*, 1998) due to its propriety of adsorbing catechols at alkaline pH and desorbing catechols at acidic pH. Alumina adsorption isolates and concentrates the low picomolar plasma concentrations of catechols into a purified low volume appropriate for injection into the HPLC apparatus. During this procedure, the alumina, at alkaline pH, is washed with water to remove other compounds and is finally acidified, desorbing the catechols into the supernatant. An aliquot of the supernatant is then injected into the HPLC apparatus. Alumina adsorption has also been used for dopamine–GSH and dopamine–cysteine adducts extraction from biological samples (Spencer *et al.*, 1998; Magnay *et al.*, 2001). However, no

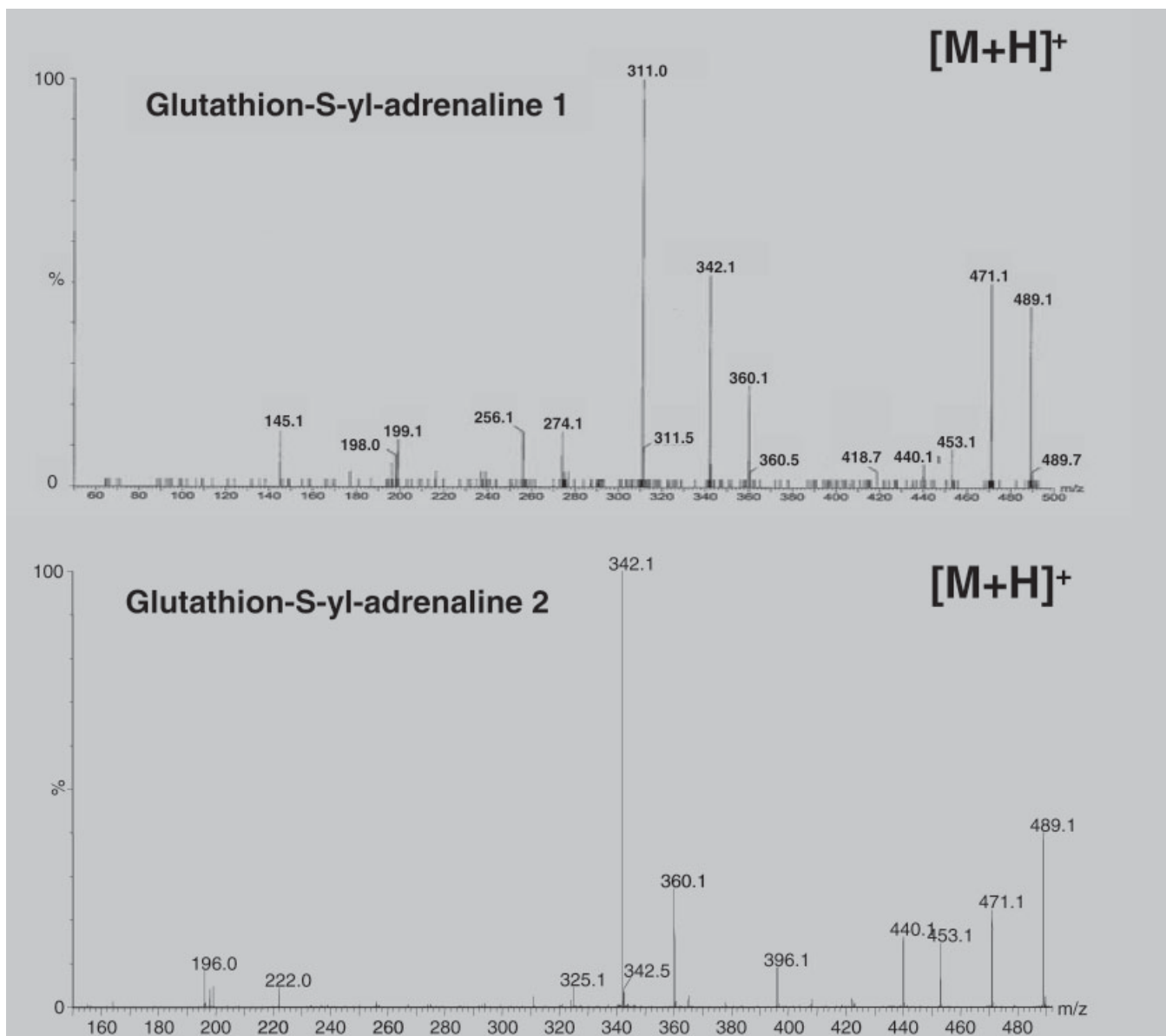


Figure 3. HPLC-ESI-MS/MS spectrum of the glutathion-S-yl-adrenaline 1 and 2 molecular ion at m/z 489.

studies have yet been done to characterize the efficacy of this extraction for adrenaline–GSH adducts or even for other catecholamine–thiol adducts.

The recovery, after alumina extraction, of GSH-adrenaline adducts from human plasma is described

in Table 1. These values compare the recovery of the adducts from plasma with and without the alumina extraction. Blank human plasma samples were previously checked to avoid interferences in the adducts retention time in the chromatograms of non-alumina

Table 1. Percentage of adrenaline-GSH recoveries in human plasma samples after alumina extraction, when compared with the same samples without alumina extraction

	Recovery (%)	
	Glutathion-S-yl-adrenaline 1	Glutathion-S-yl-adrenaline 2
15 nmol	71.4 ± 1.2	53.1 ± 1.9
3 nmol	88.5 ± 5.4	58.0 ± 1.2
0.6 nmol	93.8 ± 1.3	51.1 ± 0.4

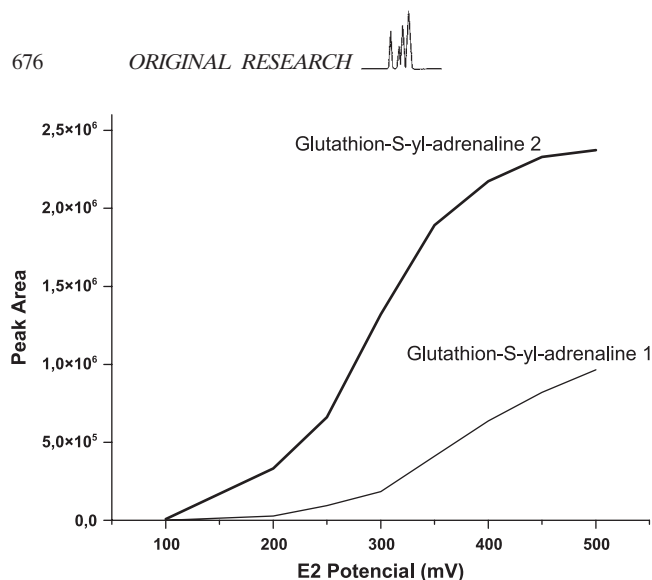


Figure 4. Hydrodynamic voltammograms for glutathion-S-yl-adrenaline 1 and 2.

extracted samples. The extraction recoveries were different for both adrenaline-GSH monoconjugated adducts. For glutathion-S-yl-adrenaline 1, recovery values vary between 71.4 and 93.8%. For glutathion-S-yl-adrenaline 2, recovery values are more consistent for the different adducts amounts and vary between 51.1 and 58.0%. Noteworthy, urine and plasma catecholamine recoveries after alumina extraction of 70–80% have been reported (Wu and Gornet, 1985; Magnay *et al.*, 2001).

Validation of the analytical procedure

The method's selectivity, detection limit, linearity, and precision (injection repeatability, procedure repeatabil-

ity, intermediate precision), and the recovery of the adrenaline-GSH adducts in human plasma after alumina extraction were evaluated.

Figure 5 shows the coulometric chromatograms obtained with human plasma samples spiked with GSH adducts after alumina extraction and blank human plasma samples with and without alumina extraction. Concerning the selectivity of the method, although some human plasma samples had no interfering peaks in the same retention time of the adducts, the majority of the tested plasma samples showed interferences in the chromatographic region of the adrenaline-GSH adducts, as can be observed in the chromatograms of the blank plasma without alumina extraction (Fig. 5). By comparing the chromatograms obtained after alumina extraction of human plasma with and without spiking with adrenaline-GSH adducts (Fig. 5), one can observe the selectivity of these peaks for the adducts. Thus, the alumina extraction is crucial to clean interferences in human plasma that might otherwise prevent the evaluation of the adrenaline-GSH adducts, especially for values that are near to the detection limits.

The detection limits for each GSH adduct in human plasma with and without alumina extraction were established as the amount of adrenaline necessary to synthesize detectable adduct peaks and are described in Table 2. The improvement of the detection limit after alumina extraction of human plasma can be observed, which is an important advantage of this extraction procedure since very low levels of these adducts in human plasma are expected. Physiologically, plasma adrenaline levels are lower than 1 nM (Goldstein *et al.*, 2003). However, these levels can significantly increase under

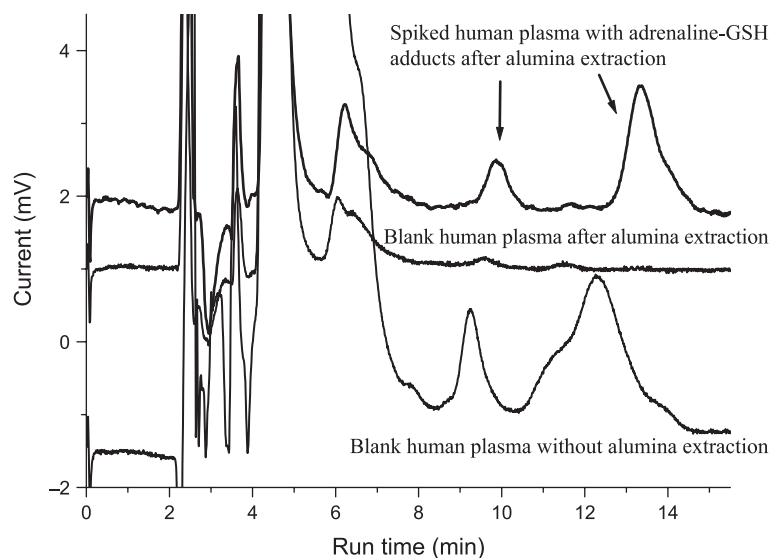


Figure 5. Coulometric chromatograms obtained after alumina extraction of human plasma samples spiked with adrenaline-GSH adducts or blank. A coulometric chromatogram of a blank human plasma sample without alumina extraction is also shown.

Table 2. Validation parameters of the analytical procedure for the determination of adrenaline–GSH adducts in phosphate buffer and human plasma with or without alumina extraction

	Without alumina extraction				With alumina extraction			
	Buffer		Plasma		Buffer		Plasma	
Glutathion-S-yl-adrenaline	1	2	1	2	1	2	1	2
Injection repeatability (CV)	1.8	2.1	3.0	1.7	1.9	0.7	1.6	1.4
Procedure repeatability (CV)	1.2	1.4	4.2	4.9	5.4	5.6	3.4	4.9
Intermediate precision (CV)	3.8	5.5	—	—	—	—	—	—
Detection limit ^a (pmol)	—	—	300	9.4	—	—	75	2.5

^a Expressed as adrenaline amount.

certain pathological conditions, such as hypoglycaemia, hemorrhagic hypotension, asphyxiation, circulatory collapse, heart ischemia, pheochromocytoma distress and drug therapy (Rupp *et al.*, 1994; Dhalla *et al.*, 1996; Akiyama and Yamazaki, 2001; Goldstein *et al.*, 2003). Plasma adrenaline levels around 11 nM in pheochromocytoma patients (Hegedus, 2000) and 20 and 58 nM in adrenaline administered patients with severe heart failure (Raymondos *et al.*, 2000) were reported. Furthermore, plasma catecholamines levels can increase as result of drug abuse. Adrenaline concentrations around 146 nM were found in rat plasma after administration of *d*-amphetamine (Carvalho *et al.*, 1997). The detection limit of 2.5 pmol of adrenaline for glutathion-S-yl-adrenaline 2 corresponds to a concentration of 5 nM of adrenaline in plasma, which is in agreement with the levels of adrenaline expected in pathological or distress situations. Noteworthy, the detection limit evaluation was performed with an injection of 20 μ L of samples in HPLC. An increase of the injection volume would improve the detection of this adducts in human plasma.

The linearity of the method was tested in quadruplicate in buffer and in plasma samples with and without alumina extraction, spiked with adducts synthesized with 0, 1, 5, 25 and 125 nmol of adrenaline. For all situations, the regression coefficient was always higher than 0.99. Linearity was also tested in triplicate after alumina extraction of plasma samples spiked with adducts prepared with adrenaline concentrations that produced values of adducts near their detection limits. Thus, for glutathion-S-yl-adrenaline 1, linearity was tested for 0, 0.15, 0.3, 0.6 and 3 nmol of adrenaline and for glutathion-S-yl-adrenaline 2 for 0, 9.4, 18.8, 37.5 and 75.0 pmol of adrenaline. In both curves the regression coefficient was higher than 0.999.

The precision of an analytical procedure is related to the random scatter of the results relative to the mean value. This parameter was characterized by the injection repeatability, procedure repeatability and intermediate precision, which are described in Table 2. According to the *Guidance for Industry: Bioanalytical Method Validation* (Food and Drug Administration,

2001), the precision determined at each level (injection repeatability, procedure repeatability and intermediate precision) should not exceed 15% of the coefficient of variation (CV), except for concentrations near the lower limit of quantification, where it should not exceed 20% of the CV. Thus, the CV values obtained (always less than 6%) show a good precision for this method, for all three evaluated parameters.

Evaluation of adrenaline–glutathione adducts stability and its spontaneous formation

Adrenaline–GSH adducts were stable in 5% perchloric acid at room temperature for eight days since the CV of adduct peaks area (in absolute values) and also the ratio between peak area adduct 2:adduct 1 for the 5 different injections performed during one week period were lower than 10%.

The possibility of spontaneous adrenaline–GSH adducts formation at room temperature in the presence of adrenaline and GSH, in Tris–EDTA buffer at pH between 8.3 and 8.6 during the 15 min required for the adsorption of the adducts in alumina, was tested. Adducts formation, without enzyme catalysis, during the alkalization step in the alumina extraction can produce an artifact, which must be avoided. In fact, the results showed a time dependent formation of these adducts. Notably, the formation of these adducts was reduced in presence of 1 mM ascorbic acid in the Tris–EDTA buffer and completely avoided by the presence of 5 mM ascorbic acid.

It was also studied the possibility of adrenaline–GSH adducts formation with adrenaline and GSH in the 5% perchloric acid. Surprisingly, it was observed the presence of adducts after 24 h of incubation at room temperature and more remarkably at -80°C . Once again, the adducts formation under those conditions was prevented by the presence of 5 mM ascorbic acid in 5% perchloric acid solution. Thus, it is crucial to use 5 mM ascorbic acid to prevent adrenaline oxidation and subsequent conjugation with GSH in all samples where adrenaline and GSH are present, even in 5% perchloric acid medium.

CONCLUSION

We report a method to evaluate the presence of adrenaline–GSH adducts in human plasma that can also be applied to other biological matrices to study the toxicity induced by the catecholamines oxidation process. The developed HPLC-ECD methodology was validated for the detection of adrenaline GSH adducts in human plasma, using adrenaline-GSH adducts extraction by alumina adsorption. The alumina extraction is important to remove interferences from the matrix. In spite of the relatively low recovery of the second adduct, the detection limit falls within the adrenaline plasma concentrations that may be expected under certain pathophysiological conditions and/or drug treatments. The obtained results also suggest that, for the application of this methodology to clinical samples, it is of utmost importance to immediately acidify the biological samples and to use 5 mM ascorbic acid, to prevent the spontaneous formation of these compounds.

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