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Determination of homovanillic acid (HVA) in human plasma by HPLC with coulometric detection and a new SPE procedure

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Abstract

A sensitive high-performance liquid chromatographic method has been developed for the determination of homovanillic acid (HVA), the main metabolite of dopamine, in human plasma. Analyses were carried out on a reversed-phase column (C8, 250 mm × 4.6 mm i.d., 5 μm) using a mobile phase composed of 10% methanol and 90% aqueous citrate buffer, containing octanesulfonic acid and EDTA at pH 4.8. Coulometric detection was used, setting the guard cell at +0.100 V, the first analytical cell at −0.200 V and the second analytical cell at +0.500 V. A careful solid-phase extraction procedure, based on strong anion exchange (SAX) cartridges (100 mg, 1 mL), was implemented for the pre-treatment of plasma samples. Extraction yield was satisfactory, being the mean value 98.0%. The calibration curve was linear over the concentration range of 0.2–25.0 ng mL^{-1} of homovanillic acid. The limit of quantitation (LOQ) was 0.2 ng mL^{-1} and the limit of detection (LOD) was 0.1 ng mL^{-1}. The method was successfully applied to plasma samples from former alcohol, cocaine and heroin addicts. Results were satisfactory in terms of precision and accuracy. Hence, the method is suitable for the determination of homovanillic acid in human plasma.

Keywords: Homovanillic acid; High-performance liquid chromatography; Coulometric detection; Human plasma; Solid-phase extraction

1. Introduction

The monitoring of catecholamines and their metabolites in human plasma is a useful clinical research tool for examining the etiologies of neuroendocrine disorders [1] and the involvement of dopaminergic and adrenergic activity under different physiological or pathological conditions [2].

3,4-Dihydroxyphenylethylamine (Dopamine) is the main central neurotransmitter involved in satisfaction and reward systems, which in turn are involved in drug self-administration phenomena [3,4]. Chronic self-administration of abuse drugs causes functional impairment in several neurostransmitter systems and in particular in the dopaminergic brain areas [5].

Homovanillic acid (HVA, 4-hydroxy-3-methoxyphenylacetic acid, Fig. 1a) is the main metabolite of dopamine and can thus be used as a marker of dopamine functioning and metabolism especially with regard to reward systems, emotional responses, personality trait expression and psychopathological phenomena [6]. Recent studies have found that HVA is a neuroendocrine marker of behavioural disorders and vulnerability to drug abuse [7–9].

Plasma HVA concentrations can be employed to assess dopamine metabolism in biomedical research in order to find the correlations between plasma monoamine functioning and different parameters of aggression [10]. Moreover, HVA levels in human blood can be used as marker for neuroblastoma [11] and they have been found to be elevated in plasma of patients with chronic schizophrenia treated with atypical antipsychotic drugs [12–14]. The study of HVA plasma level variability is therefore very important for the diagnosis of disorders and behavioural problems [15]; consequently, there is a constant strive for the development of reliable, sensitive and precise methods for the determination of this compound in plasma samples.

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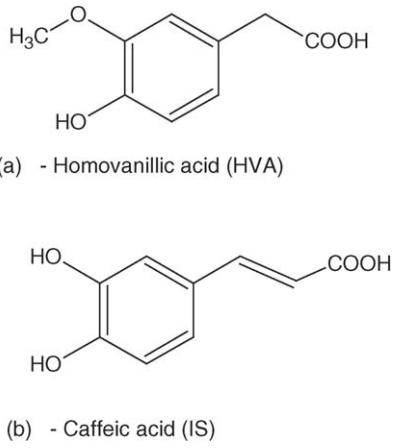

Fig. 1. Chemical structures of (a) homovanillic acid and (b) caffeic acid (IS).

Several analytical methods have been published for the determination of HVA in different biological matrices including urine [16,17], cerebro-spinal fluid [18], brain tissues [19], animal plasma [20,21] and other tissues [22]. Other methods regard the analysis of this compound in human plasma; the vast majority of them are based on the use of HPLC with electrochemical [15,21,23–29] detection, however at least one uses HPLC with fluorescence detection [17] and another capillary gas chromatography–mass spectrometry [30].

HPLC with electrochemical detection seems to be particularly suitable for the assay of HVA, due to its high sensitivity and selectivity and to the electroactivity of the analyte. In fact, the amounts to be determined in plasma are in the submicroanalysis range [31]. This is a difficult analytical problem, especially if one takes into account the chemical instability of HVA and the complex biological matrix from which it has to be extracted.

Among the available papers which report the analysis of HVA in human plasma, some are quite laborious, requiring the use of two different solid-phase extraction cartridges [23,29] or analyte derivatisation [17]; other authors use very expensive instrumentations such as mass spectrometry [30] or microbore HPLC [21,25]. Other reported methods are not completely satisfactory: they use high volumes of plasma (1 mL) and have high analytical variability (coefficient of variation up to 10%) [26], or have not satisfactory sensitivity (LOD = 2.0 ng mL^{-1}), probably due to the use of protein precipitation [15,28] which introduces a dilution step into the procedure.

The aim of this study is the development of a selective and sensitive method for evaluating HVA levels in human plasma. The proposed method is based on the use of high-performance liquid chromatography with coulometric detection. An original procedure of plasma pre-treatment based on solid-phase extraction (SPE) has been implemented and has provided high extraction yields. The method has successfully been applied to the determination of HVA levels in human plasma. This analytical paper is part of a broader interdisciplinary research, with the aim of studying neurobiological correlates of behaviour [32,33] and developing new strategies for the prevention and the therapy of drug addiction [34].

2. Experimental

2.1. Chemicals

Homovanillic acid (4-hydroxy-3-methoxy phenyl acetic acid, HVA), caffeic acid, used as the Internal Standard (IS, Fig. 1b) for the control of retention times only, and bovine albumin were purchased from Sigma Chemicals (St. Louis, MO, USA).

Citric acid, ethylendiaminotetracetic acid sodium salt (EDTA), sodium metabisulfite, 30% (w/w) concentrated ammonia, 37% (w/w) hydrochloric acid, 2 M sodium hydroxide, sodium chloride (NaCl), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate anhydrous (Na_2HPO_4) and methanol were analytical grade from Carlo Erba (Milan, Italy).

The 1-octanesulfonic acid monohydrate sodium salt (OSA) was from Fluka Biochemika (Buchs, Switzerland).

Ultrapure water (18.2 MΩ cm) from a MilliQ apparatus by Millipore (Milford, MA, USA) was used.

2.2. Apparatus and chromatographic conditions

The HPLC apparatus consisted of a Jasco (Tokyo, Japan) PU-1580 chromatographic pump and an ESA (Milford, MA, USA) Coulochem III coulometric detector, equipped with cells having porous graphite working electrodes and α-hydrogen/palladium reference electrodes. The conditioning cell was set at +0.100 V; in the analytical cell, detector 1 was set at −0.200 V and detector 2 at +0.500 V, with a range of 1 μA and an output of +1.00 V. The chromatograms were monitored at the analytical detector 2.

Data were handled by means of Software Cromatography Station (CSW 32 v. 1.4) from DataApex (Prague, Czech Republic).

The chromatographic separation was achieved by isocratic elution on a Varian (Harbor City, CA, USA) Microsorb reversed-phase column (C8, 250 mm × 4.6 mm i.d., 5 μm), with a Phenomenex Security Guard precolumn (C8, 4.0 mm × 3.0 mm i.d.).

The mobile phase was a mixture (10:90, v/v) of methanol and an aqueous solution of 49 mM citric acid, 110 mg L^{-1} octanesulfonic acid and 22 mg L^{-1} EDTA adjusted to pH 4.8 with 2 M sodium hydroxide.

The flow rate was 1.2 mL min^{-1} and the samples were injected by means of a 20 μL loop.

Prior to use, the mobile phase was filtered through Varian nylon filters (47 mm diameter, 0.2 μm pore size) and degassed by sonication.

2.3. Solutions

The stock solutions of HVA were 1 mg mL^{-1} and were prepared by dissolving 20 mg of the substance in 20 mL of ultrapure water to which 20 mg of sodium metabisulfite, 160 mg of sodium chloride and 10 μL of concentrated hydrochloride acid (37%) were previously added. This saline solution prepared at acidic pH and containing an antioxidant is useful to avoid analyte degradation.

The stock solutions of IS were 1 mg mL^{-1} and were prepared fresh every 2 days by dissolving 20 mg of caffeic acid in 20 mL of methanol.

Standard solutions were prepared daily by diluting the stock solutions with the mobile phase.

"Reconstituted" or blank plasma was prepared by dissolving 160 mg of NaCl, 23 mg of Na_2HPO_4, 4 mg of KH_2PO_4, 4 mg of KCl and 800 mg of bovine albumin in 20 mL of ultrapure water.

2.4. Plasma sample collection

The study was carried out in collaboration with the Ser.T. (Servizio Tossicodipendenze, Drug Addiction Centre) of Roma, directed by Dr. Claudio Leonardi, on plasma samples from former drug users. The blood samples (3 mL each) were drawn into test tubes containing anticoagulant; after centrifugation, the supernatant plasma was transferred into test tubes and frozen at −80 °C until the analysis.

2.5. Sample pre-treatment: SPE procedure

Analytichem International (Harbor City, CA) BondElut SAX cartridges (100 mg, 1 mL) were used to extract the analytes from plasma with a SPE procedure. Cartridges were activated and conditioned with 5× 1 mL of methanol and 5× 1 mL of water. Fifty microliters of the IS (50 ng mL^{-1}) were added to 250 μL of plasma and the mixture was diluted with 500 μL of water and loaded onto the previously conditioned cartridge. After washing with 1 mL of water and 1 mL of methanol, the cartridge was dried applying full vacuum (−30 kPa) for 1 min using a Vac Elut (Varian) apparatus. The elution of the analytes was carried out with 1 mL of 5% ammonia solution in methanol, applying full vacuum again for 1 min. The eluate was brought to dryness by rotary evaporator, and the residue was re-dissolved in 125 μL of mobile phase.

2.6. Method validation

2.6.1. Linearity

Standard solutions at different concentrations of HVA in the 0.2–25.0 ng mL^{-1} range were added to 250 μL of blank plasma and, after the SPE procedure, analysed in triplicate. The analyte peak areas were plotted against the corresponding concentrations and the 10-point calibration curves were set up by means of the least-square method.

2.6.2. Extraction yield (absolute recovery)

Analyte standard solutions at three different concentrations of HVA were added to 250 μL of blank plasma (in order to obtain final analyte concentrations after SPE of 0.4, 25.0 and 50.0 ng mL^{-1}), subjected to the SPE procedure and injected into the HPLC.

The analyte chromatographic peak areas thus obtained were compared to those obtained from standard solutions at the same concentration, and the percentage extraction yield was calculated.

2.6.3. Precision

The blank plasma was spiked with HVA at three concentration levels to give final concentrations after SPE of 0.4, 25.0 and 50.0 ng mL^{-1}, corresponding to the lower, middle and upper limit of the calibration curves. After thorough mixing, extraction and HPLC analysis were then performed. The procedure was repeated at least six times within the same day to obtain the repeatability, and over different days to obtain the intermediate precision.

2.6.4. Accuracy

Analyte standard solutions at three different concentrations (in order to obtain final analyte additions of 5.0, 100 and 300 ng mL^{-1}) were added to 250 μL of plasma and the mixture subjected to the SPE procedure described above (Section 2.5). Recovery values were calculated according to the following formula: 100 × ([after spiking] − [before spiking])/[added].

3. Results and discussion

3.1. Choice of the chromatographic conditions

Since HVA is an electroactive compound, detection by means of a coulometric system can be an attractive choice, in that it grants high sensitivity and selectivity at lower costs than similarly sensitive detectors such as mass spectrometers.

Some chromatographic parameters such as mobile phase and electrochemical conditions were investigated to obtain a good separation of the analyte from the IS and from possible interference of the biological matrix, while maintaining acceptable analysis times.

The chromatographic conditions already used in our previous papers on the determination of catecholamines [35–37] were chosen as the starting conditions for the analysis of HVA, which has chemical–physical properties similar to those of catecholamines. Using a C8 Microsorb reversed-phase column (250 mm × 4.6 mm i.d., 5 μm), different modifications to the mobile phase were investigated in order to obtain shorter retention times. The percentage of methanol between 5.0% and 20.0% and the pH value in the 3.0–5.0 range were tested. Good results in terms of run times and resolution were obtained with a mobile phase consisting of a mixture (10:90, v/v) of methanol and a 49 mM, pH 4.8 citrate buffer containing 110 mg L^{-1} of octanesulfonic acid and 22 mg L^{-1} of EDTA, flowing at 1.2 mL min^{-1}. Under these conditions, HVA is eluted at 8.3 min, while caffeic acid, used as the internal standard for the control of retention times, has a retention time of 11.2 min. A chromatogram of a standard solution containing HVA (25 ng mL^{-1}) and the IS (20 ng mL^{-1}) is shown in Fig. 2.

Several trials were carried out to find the best electrochemical conditions of the coulometric detector, in terms of sensitivity and selectivity. Potential values ranging from −0.100 to −0.600 V for E_1 and from +0.100 to +0.600 V for E_2 were tried. A reduction potential of −0.200 V was chosen for E_1, while an oxidation potential of +0.500 V was chosen for E_2, which give a good sensitivity while obtaining a satisfactory cut-off of biological interference.

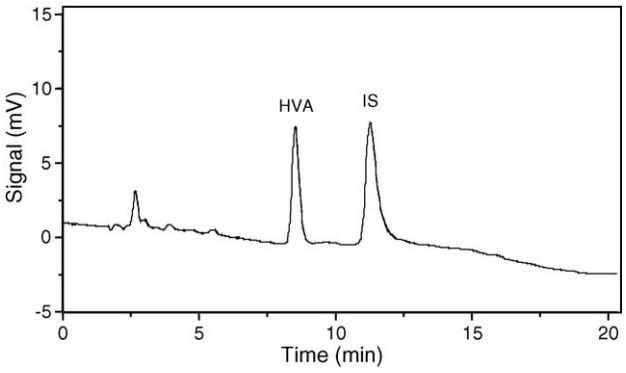

Fig. 2. Chromatogram of a standard solution containing 25 ng mL^{-1} of HVA and 20 ng mL^{-1} of the IS.

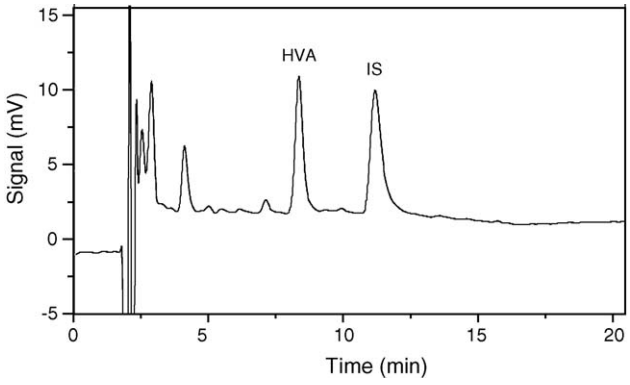

Fig. 3. Chromatograms of a blank plasma sample spiked with 25 ng mL^{-1} of HVA and 20 ng mL^{-1} of the IS.

3.2. Analysis of standard solutions

Good linearity ($r^2 = 0.9999$) was obtained over the HVA concentration range: 0.4–50.0 ng mL^{-1}. Precision assays carried out at three different levels (0.4, 25.0 and 50.0 ng mL^{-1}) also gave satisfactory results: the relative standard deviation (R.S.D.%) of repeatability (intraday precision) was equal to or lower than 3.8%. Intermediate (interday) precision was satisfactory, with R.S.D. values always lower than 3.9%. The limit of quantification (LOQ) and the limit of detection (LOD) were 0.4 and 0.2 ng mL^{-1}, respectively.

3.3. Development of the solid-phase extraction procedure

In order to improve the selectivity and reproducibility of the method, a new plasma pre-treatment procedure by solid-phase extraction was implemented. Different sorbents were tested for SPE, such as hydrophilic–lipophilic balance (HLB), lipophilic (C8, C18), weak anionic exchange (diethylaminopropyl, DEA; primary–secondary amine, PSA) and strong anionic exchange (trimethylaminopropyl, SAX). The extraction yields obtained using HLB, DEA and PSA cartridges were rather low (<50%), while C8 and C18 cartridges gave rise to heavy intereference in the chromatogram. Best results were obtained using SAX cartridges, which were thus chosen for all subsequent assays.

All the steps of loading, washing and elution were carefully examined. Several kinds of loading step were tested: different buffers in the 2.5–8.0 pH range and, finally, water, which turned out to be the best choice. The optimum loading condition was a 250 μL plasma aliquot, diluted 1:2 with water.

If the washing step was carried out with basic or acidic water/methanol mixtures it resulted in a sharp drop in analyte recovery, while other kinds of washing solutions (water alone, different kinds of buffers) prevented the satisfactory purification of the samples. The best results were obtained when washing the cartridge with 1 mL of water followed by 1 mL of methanol.

With regard to the elution step, methanol or acidic methanol/water mixtures did not give satisfactory results in terms of analyte recovery. On the contrary, a 5% ammonia solution in methanol gave good extraction yields, and this was chosen as the elution solution. The eluate was then dried under vacuum and redissolved in 125 μL of mobile phase.

In conclusion, when the above outlined SPE procedure is carried out on blank plasma samples the chromatograms do not show any interference from the matrix and the analyte, which is concentrated two times with respect to the original plasma sample, is detected as a neat peak. As an example, the chromatogram of a blank plasma sample spiked with HVA is reported in Fig. 3.

3.4. Method validation

Having thus assured the suitability of the SPE procedure, calibration curves were set up on blank plasma by adding to the plasma aliquots of standard solutions of the analyte at different concentrations and the IS and subjecting the resulting mixture to the SPE procedure.

Calibration curves were set up in the 0.2–25.0 ng mL^{-1} range (corresponding to 0.4–50.0 in the injected solutions, respectively) for HVA. Good linearity ($r^2 = 0.9993$) was found in this range, with regression equation $y = 0.02008 + 0.04021x$, where y is the peak area, expressed as mV s and x is the compound concentration, expressed as ng mL^{-1}.

The LOQ was 0.2 ng mL^{-1}, while the LOD was 0.1 ng mL^{-1} (corresponding to 0.4 and 0.2 ng mL^{-1} in the injected solutions, respectively). Both values were calculated according to the United States Pharmacopeia [38] and "Crystal City" guidelines [39]. Extraction yield (absolute recovery) and precision assays were carried out on reconstituted plasma spiked with analyte concentrations corresponding to the lower, middle and upper limit of the calibration curves. The results of these assays are reported in Table 1.

As one can note, mean extraction yields were always higher than 97.6%. Precision results were also satisfactory: R.S.D. values for repeatability were always lower than or equal to 3.9%; R.S.D. values for intermediate precision were lower than or equal to 4.0%.

3.5. Method selectivity

Selectivity was evaluated by injecting into the HPLC standard solutions of several drugs, mainly Central Nervous System drugs: antipsychotics, antidepressants and mood stabilizers. The complete list of these drugs is reported in Table 2. As can

Table 1
Extraction yield assays

Compound	Concentration (ng mL^{-1})	Extraction yield (%)[a]	Repeatability, R.S.D.%[a]	Intermediate precision, R.S.D.%[a]
HVA	0.4	97.8	3.9	4.0
	25.0	97.6	3.7	3.9
	50.0	98.6	2.9	3.1

[a] $n = 6$.

be seen, only four of the tested drugs (levodopa, carbidopa, metamphetamine and 3,4-methylenedioxymethamphetamine or ecstasy) are revealed within a 30-min chromatographic run, and none interferes with the analysis. Thus, selectivity was deemed satisfactory.

3.6. Analysis of patient plasma samples

Having thus validated the method, it was successfully applied to the analysis of HVA in plasma of several subjects who were former alcohol, cocaine or heroin addicts, as well as some controls. A chromatogram of a plasma sample from a former cocaine user undergoing psychotherapy is reported in Fig. 4. As can be seen, the sample pre-treatment allows to obtain chromatograms devoid of interference from the biological matrix. HVA is detected as a neat peak at 8.3 min; by interpolating the peak area on the calibration curve, a concentration of 13.2 ng mL^{-1} was obtained, which corresponds to a concentration of 6.6 ng mL^{-1} in the original plasma sample.

Table 2
Drugs tested for interference

Therapeutic class	Compound	t_R (min)
Antiparkinson	Biperiden	n.d.[a]
	Carbidopa	3.0
	Levodopa	2.5
Antidepressants	Fluoxetine	n.d.
	Fluvoxamine	n.d.
	Imipramine	n.d.
	Paroxetine	n.d.
	Sertraline	n.d.
Antipsychotics	Clozapine	n.d.
	Fluphenazine	n.d.
	Risperidone	n.d.
Anxiolytics-hypnotics	Brotizolam	n.d.
	Diazepam	n.d.
	Flurazepam	n.d.
	Lorazepam	n.d.
Antiepileptics	Lamotrigine	n.d.
	Levetiracetam	n.d.
	Primidone	n.d.
	Phenytoin	n.d.
	Ethosuximide	n.d.
Antihistaminics	Promethazine	n.d.
	Triprolidine	n.d.
Abuse drugs	Amphetamine	n.d.
	Metamphetamine	2.7
	Ecstasy	2.8
	Δ9-Tetrahydrocannabinol	n.d.

[a] n.d.: not detected within a 30-min chromatographic run.

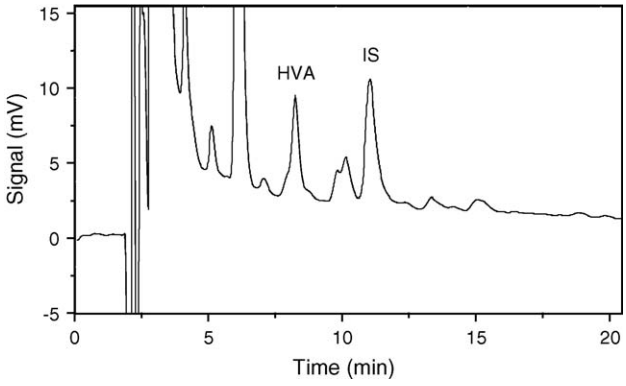

Fig. 4. Chromatogram of a plasma sample from a former cocaine user undergoing psychotherapy.

Method accuracy was evaluated by means of recovery studies at three different concentration levels ($n = 3$ for each level), as reported in Section 2.6.4.

Results were very satisfactory: mean recovery values were 97.0% at 5.0 ng mL^{-1}, 96.0% at 10.0 ng mL^{-1} and 93.4% at 30.0 ng mL^{-1}. Standard deviation values were also good: 3.0, 2.8 and 2.2, respectively.

4. Conclusion

The method developed for the determination of homovanillic acid in human plasma, based on HPLC with coulometric detection, has various advantages: high precision and accuracy, good selectivity and feasibility and, above all, high sensitivity due both to the electrochemical detection and to the original SPE procedure. In fact, the SPE step carried out by means of SAX cartridges has allowed to obtain very high extraction yields (>97%) and good selectivity (no interference from either the endogenous matrix or several tested drugs) and an improvement of sensitivity (due to the fact that the analyte is concentrated two times with respect to the original plasma sample).

When compared to other methods found in the literature, the present method has the advantage of being inexpensive since it does not require expensive instrumentation [21,25,30] and feasible since it does not need complicated sample pre-treatments [23,29] or laborious analyte derivatisation [17].

In conclusion, the proposed HPLC method is suitable and advantageous for the reliable analysis of HVA in human plasma, and it has been successfully applied to the determination of the analyte in samples from former drug addicts subjected to detoxification treatment [40].

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