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Stability of apomorphine in plasma and its determination by high-performance liquid chromatography with electrochemical detection

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Abstract

Dilute solutions (50 ng/ml) of apomorphine in plasma are unstable at 37°C and pH 7.4. The chemical half-life is only 39 min. Mercaptoethanol (0.01%) is effective in stabilizing these samples while sodium metabisulphite (1%), which is generally used, is not effective. Biological samples are extracted with diethyl ether (recovery 96.5%) and analysed using HPLC with coulometric detection (oxidation potential 0.25 V). The stationary phase employed was C_{18} material (4 μm) and the mobile phase was phosphate buffer (pH 3)–acetonitrile (70:30, v/v). The flow-rate was 1.8 ml/min. This bioanalytical method presents a reliable tool for pharmacokinetic studies in man.

1. Introduction

Apomorphine is a long-used drug which has recently received renewed attention because it has been found to have beneficial effects on Parkinsonian patients who experience "on" and "off" disabling problems after prolonged use of levodopa [1–7].

A number of analytical methods have been developed for the determination of apomorphine in biological samples including gas chromatographic [8], spectrophotometric [9], fluorimetric [10], mass fragmentographic, [11] radioenzymatic [12], and radiochemical methods [13]. Recently high-performance liquid chromatographic (HPLC) methods coupled with ultraviolet [14,15], fluorimetric [16] and electrochemical detection (ED) [17–20] have been reported. Enzymatic radioisotopic methods and HPLC–ED are the most sensitive methods with detection limits of 0.8 ng/ml and 0.3–0.5 ng/ml respectively.

Several methods have been explored for the extraction of apomorphine from biological samples using different solvents for direct extraction or after formation of a complex with the diol group [19]. However, most of the known sample processing methods are either complex, unable to avoid interference, or result in a concentration step that is not sufficient to attain the required sensitivity.

None of the analysis methods published so far addressed the problem of the stability of apomorphine in biological samples in a quantitative manner. Apomorphine is a very labile compound in solution. Aqueous solutions oxidize in

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light and air, turning into green. The rate of degradation is pH dependent, stability decreasing with increasing pH [21]. In order to warrant stability of apomorphine during sample processing and during the subsequent steps of the analysis procedure, one may think of performing the assay at low pH and low temperature, of eliminating and complexing the oxidizing agents and promoters and of adding reducing agents like ascorbic acid, sodium metabisulphite, mercaptoethanol or dithiothreitol. There are no quantitative data describing the validity of a stabilization protocol for apomorphine in human plasma samples and therefore one can not rule out the possibility that stability and reproducibility problems have been biasing pharmacokinetic data.

The present paper describes the stability of apomorphine under various conditions. The influence of different stabilizers and extraction conditions have been investigated. An HPLC–ED analysis method for apomorphine has been worked out and applied to the analysis of plasma samples obtained from one patient after subcutaneous administration of apomorphine hydrochloride.

2. Experimental

2.1. Chemicals

Acetonitrile was purchased from Carlo Erba (Milano, Italy), sodium dihydrogenphosphate from Merck (Darmstadt, Germany), and diethyl ether from UCB (Leuven, Belgium). Apomorphine hydrochloride (APO-HCl) was obtained from Federa (Brussels, Belgium) and the internal standard N-propyl-norapomorphine (NPA) from Research Biochemicals (Natick, USA). All reagents were analytical grade and were used as received. The water used throughout was purified by a Millipore Milli-Q system (Bedford, USA).

The mobile phase was freshly prepared daily, filtered through a 0.45-μm filter and then degassed in ultrasonic bath for 5 min.

Standard solutions of apomorphine hydrochloride salt and internal standard were prepared in 0.1 *M* HCl at a concentration of 250 μg/ml and 1 mg/ml respectively. These solutions were kept at −40°C. No degradation occurred under these circumstances, during the course of the study.

2.2. Instrumentation

HPLC analyses were carried out with a chromatographic system consisting of a Model 6000A pump, connected to a type U6K injector from Waters Associates (Milford, USA). The detection system consisted of a Model 5100A coulometric electrochemical detector from Environmental Sciences Associates (Bedford, USA) using a Model 5010 dual-electrode analytical cell and a Model 5020 guard cell. The cells were protected by carbon prefilters from Environmental Sciences Associates (Bedford, USA).

The output of the detector was a 0–1 V signal which was channelled to a computer terminal equipped with Kontron PC Integrator software (version 2.60) for data recording and processing.

2.3. Chromatographic conditions

Chromatographic separation was carried out using a cartridge placed under radial compression (10 cm × 8 mm I.D.) filled with reversed-phase C_{18} material (Nova-Pack) of particle size 4 μm from Waters Associates (Milford, USA). The analytical column was protected by a protective guard-pak cartridge (Novapak C_{18}) from Waters Associates.

Separation was carried out at 20–22°C. The mobile phase consisted of acetonitrile–phosphate buffer (NaH_2PO_4 0.01 *M*, EDTA 1 m*M*, pH 3) (30:70, v/v). The flow-rate was 1.8 ml/min. The detection of apomorphine and the internal standard was carried out in the oxidative mode at an operating potential of 0.25 V. The guard cell placed before the injector was operated at a potential of 0.30 V.

2.4. Sample processing

Samples (0.5 ml) of stabilized plasma (as outlined below) were spiked with APO-HCl and/or NPA and extracted with diethyl ether (2.5

ml). The mixture was vortex-mixed for 30 s, and centrifuged for 5 min at 900 *g*. The diethyl ether layer (2.0 ml) was evaporated in ca. 15 min to dryness under a stream of air. The samples were reconstituted in 0.5 ml of the mobile phase and aliquots of 50 μl were injected onto the HPLC system. When performing the analysis of plasma from patients treated with apomorphine, the samples were reconstituted in 0.1–0.5 ml of mobile phase in order to introduce a concentration step where required.

3. Results and discussion

3.1. Voltammogram and chromatogram

Chromatograms of a blank plasma (a), plasma spiked with APO-HCl 50 ng/ml and NPA 100 ng/ml (b), and a plasma sample obtained from a patient (c) are presented in Fig. 1. There was no interference from endogenous plasma compounds.

The operating voltage was determined by a stepwise increase in voltage applied to the detection cell and recording the corresponding peak area after repeatedly injecting 2.5 ng of apomorphine hydrochloride and 5 ng of the internal standard in 50 μl of mobile phase. A voltammogram was constructed from these data by plotting the peak areas as a function of the applied voltage (Fig. 2). For optimum operating conditions, the operating potential was set at 0.25 V. The guard cell placed before the injector was operated at a potential of 0.30 V.

3.2. Stability of apomorphine in plasma

The stability of apomorphine was investigated at pH 7.4, and temperatures of 37°C, 20°C and 0°C. This was achieved by taking 5 ml of plasma adjusted to pH 7.4, equilibrating it to the desired temperature, and then spiking it with apomorphine hydrochloride to a concentration of 50 ng/ml. Aliquots of 500 μl were then processed and analyzed as a function of time. Fig. 3 shows the degradation profiles as a decrease in apomorphine chromatographic peak area as a function of time elapsed between spiking and processing

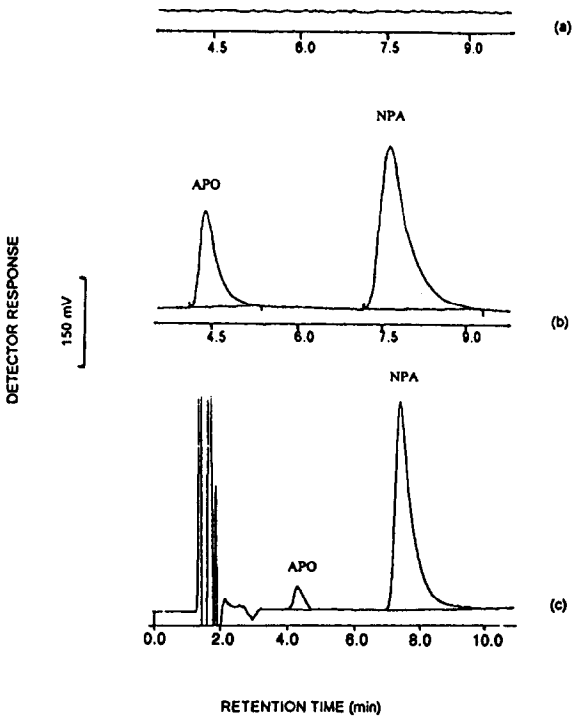

Fig. 1. Chromatogram obtained after processing (a) blank plasma, (b) plasma spiked with 50 ng/ml apomorphine hydrochloride (APO) and 100 ng/ml of the internal standard (NPA), and (c) a plasma sample obtained from a patient 15 min after receiving 3.5 mg of apomorphine hydrochloride subcutaneously.

of the samples. Degradation at time zero is already quite pronounced, indicating that any stabilizing protocol must start at time zero to

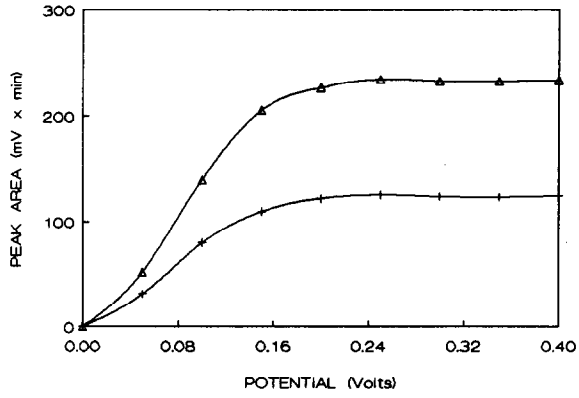

Fig. 2. Hydrodynamic voltammogram of apomorphine (×) and the internal standard N-propylnorapomorphine (△).

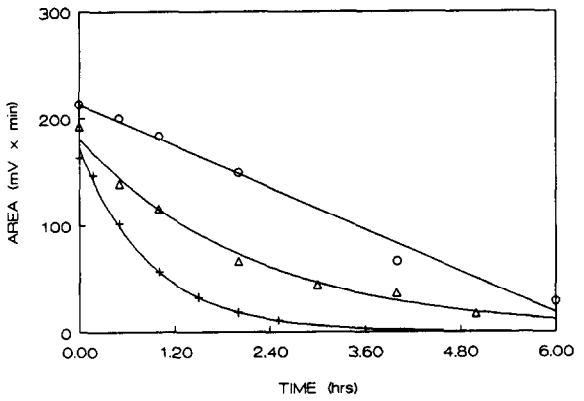

Fig. 3. Stability of apomorphine (50 ng/ml) in plasma at pH 7.4 and temperatures of 0°C (○), 20°C (△), and 37°C (×). The upper curve was fitted as a linear decay, while the lower curves were fitted as a first order decay.

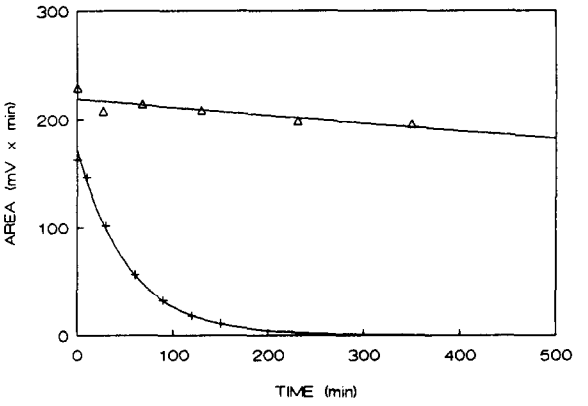

Fig. 4. Stability of apomorphine in plasma at pH 7.4 and 37°C at concentrations of 2500 ng/ml (△) and 50 ng/ml (×). The upper curve was fitted as a zero order decay, while the lower curve was fitted as a first order decay.

avoid degradation taking place during blood collection, plasma separation and sample processing. The logarithmic plot of the data at pH 7.4 and a temperature of 37°C is a straight line (first order kinetics) with a degradation constant of 0.0179 per min. and a half-life of 38.9 min. No data could be found in the literature for comparison, but the degradation rate we observed is much higher than those reported in buffer at pH 6.8 and 30°C, [21] and in rabbit urine at pH 7.6 and 37°C [22]. These discrepancies could not be attributed to the change in pH or temperature, but the concentration we used was much lower (50 ng/ml vs. 500 000 ng/ml) in order to be consistent with the expected plasma levels. This parameter was tested by repeating the same procedure in plasma respectively at a high concentration (2500 ng/ml) and a low concentration (50 ng/ml). Results are summarized in Fig. 4. We also tested the stability of apomorphine in phosphate buffer versus plasma at 50 ng/ml, pH 7.4 and 20°C. Results are summarized in Fig. 5. At a concentration of 2500 ng/ml in plasma, apomorphine was significantly more stable than at 50 ng/ml.

The order of the reaction is zero at a concentration of 2500 ng/ml as compared to first order at 50 ng/ml. Apomorphine was also slightly more stable in buffer, as compared to plasma. It was concluded that the apomorphine stability is concentration dependent and therefore stabilization strategies developed for concentrated solutions and pharmaceutical preparations need to be reevaluated and quantified when dealing with dilute biological fluids. Secondly, metabolic studies carried out in animals [23–27] at a dose 300 times that given to man may not represent a true reflection of the disposition of apomorphine in humans. Autooxidation could play a very significant role in the disposition of apomorphine from the body when therapeutic concentrations are taken into account.

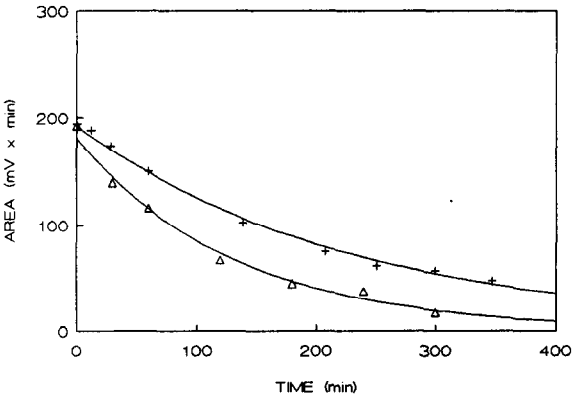

Fig. 5. Stability of apomorphine in plasma (△) and buffer (×) both at pH 7.4, 20°C and a concentration of 50 ng/ml. Both curves were fitted as a monoexponential decay.

3.3. Stabilization of apomorphine in plasma

Various antioxidants at different concentrations were investigated to evaluate their effect on the stability of apomorphine at various temperatures. The results obtained with sodium metabisulphite (1% w/v), the recommended concentration in pharmaceutical preparation and the commonly used stabilizer, and mercaptoethanol (0.01% v/v) are reported here. After addition of the stabilizers, the same procedure for further analysis was followed as described above. The effect of sodium metabisulphite was measured at 20°C and that of mercaptoethanol at 20°C, 0°C and −40°C and compared with unstabilized plasma at 20°C. Fig. 6 shows the decay of the apomorphine peak area as a function of time. At time zero the degradation of apomorphine in unstabilized plasma and in plasma with sodium metabisulphite is instantaneous, amounting to 16% and 7% respectively assuming that the peak area obtained by processing plasma stabilized with mercaptoethanol at 20°C and at time zero is 100% that of the control. Thereafter the rate of degradation is still high except for samples stabilized with mercaptoethanol. Mercaptoethanol was effective at concentrations as low as 0.001% v/v. At concentrations higher than 0.09% v/v, there was an interference with the detector response. Mercaptoethanol at a concentration of 0.01% was considered optimal with respect to possible variations in sample volumes, and in view of the possibility to concentrate samples without introducing interference with the detector response. In our pharmacokinetic study, patient samples were concentrated up to five times. From this study it is evident that apomorphine stabilization is mandatory and that any kinetic study should be carried out in a way ensuring maximum stability. This can be achieved through addition of mercaptoethanol (0.01% v/v) and working at lower temperatures (ice bath) during sample processing. Long term storage should occur at −40°C. A stability test carried out on samples stored at this temperature for three weeks did not show any changes in apomorphine content. When samples are obtained from patients, the blood should be collected in tubes already containing EDTA and 10 μl of a 1% (v/v) solution of mercaptoethanol in saline per ml of sample; the samples should be immediately placed in ice, separated and then processed or frozen.

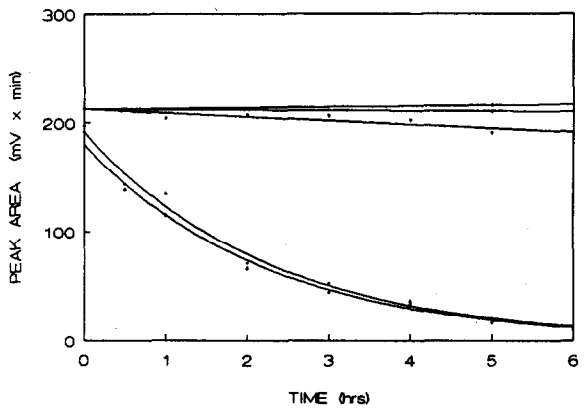

Fig. 6. Stability of apomorphine (50 ng/ml) in plasma at pH 7.4, plotted as a function of time. The three upper curves resulted from apomorphine stabilized with mercaptoethanol 0.01% v/v at −40°C (upper), 0°C (middle) and at 20°C (lower). The decay was linear with time. The lower two curves are from apomorphine stabilized with sodium metabisulphite 1% w/v at 20°C (upper) and plasma without stabilizer at 20°C (lower). Data points were fitted as a first order decay.

3.4. Recovery, inter-day and intra-day variations, bias and linearity

The recoveries of apomorphine and the internal standard were determined from the ratios of peak areas obtained after processing spiked samples to those of a reference solution, injected directly, containing the same amount of APO (50 ng/ml) or NPA (100 ng/ml) in mobile phase. Extraction using diethyl ether performed best, as compared to dichloromethane, ethylacetate, chloroform, and hexane. Evaporation of the organic phase under a stream of air yielded recoveries equal to those obtained by vacuum drying or a stream of nitrogen. The stability of apomorphine when dried in a stream of air could be due to stabilization of apomorphine in diethyl ether [28]. It is also possible that co-extracted

mercaptoethanol confers extra protection in a stream of air. At a pH of 7.4 recovery was 96.6 ± 1.5%, for apomorphine, and 99.2 ± 1.1% for NPA (mean ± S.D; $n = 10$). Although its pK_a is 7.2, apomorphine extraction was not affected by pH in the range 6.5–8.6. Thus there was no need for pH adjustment of samples collected for pharmacokinetic studies.

The intra-day ($n = 6$) and inter-day ($n = 5$) reproducibility and the bias of the assay were established at concentrations of 2.5, 12.5 and 25 ng/ml of apomorphine hydrochloride and 50 ng/ml of the internal standard in stabilized plasma. Table 1 shows the intra-day and inter-day variations and the accuracy of the method expressed as the relative standard deviation (R.S.D.) and percentage bias, respectively. The maximum variation was 6.3% and maximum bias was 5.3%

A calibration curve was established by spiking stabilized plasma samples with apomorphine hydrochloride salt over the concentration range 0.781–100 ng/ml (0.67–85.45 ng/ml apomorphine base). The internal standard was kept at a fixed concentration of 100 ng/ml. The calibration curve established by plotting peak-area ratios of apomorphine over internal standard against apomorphine base concentration resulted in a straight line almost through the origin ($y = 0.00061 + 0.01009x$, $r^2 = 0.999937$). The detection limit was estimated to be 0.67 ng/ml of apomorphine base based on a signal-to-noise ratio of 3.

Table 1
Intra-day and inter-day variation calculated as relative standard deviation (R.S.D.) and accuracy measured as % bias.

Sample	Concentration added (ng/ml)		
	2.5	12.5	25.0
Intra-day (n = 6)			
Mean	2.569	12.108	24.840
S.D.	0.162	0.310	0.777
R.S.D.	6.32	2.56	3.13
Bias	2.76	−3.13	0.6
Inter-day (n = 5)			
Mean	2.632	112.369	25.492
S.D.	0.200	0.309	0.497
R.S.D.	4.240	2.50	1.95
Bias	5.28	−1.80	1.96

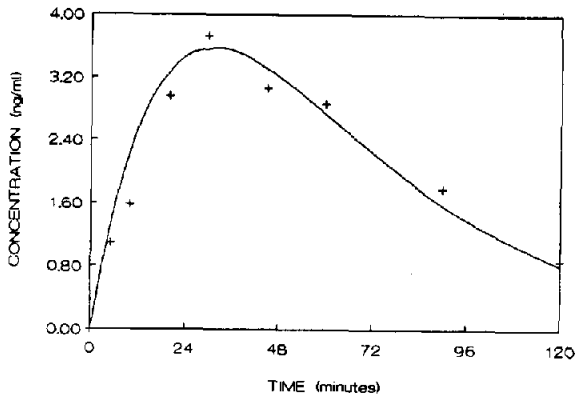

Fig. 7. Plasma concentration–time profile of apomorphine after a 2-mg subcutaneous dose of apomorphine hydrochloride.

3.5. Pharmacokinetic study

The applicability of the HPLC–ED analysis and the sample processing procedure has been demonstrated by studying the kinetic profile of apomorphine after subcutaneous administration of 2 mg of apomorphine HCl to a patient suffering from parkinsonism. Blood was processed and analysed as described above. Blood samples obtained from patients prior to apomorphine administration did not show interference with the detector response from other concurrently administered drugs like L-dopa. Fig. 7 shows the plasma concentration–time profile for a 2-h period after administration. Apomorphine was rapidly absorbed with an absorption half-life of 15 min. The maximum plasma concentration of 3.7 ng/ml was observed 30 min after injection. The best fit of the experimental data was obtained by using a 1 + 1 open compartmental model. The terminal elimination half-life was 32.5 min and the apparent volume of distribution (area) was 265 l.

4. Conclusion

The described method for the stabilization and extraction of apomorphine in human plasma is highly selective and shows excellent recovery and reproducibility. Its usefulness in pharmacokinetic studies of apomorphine in humans has been demonstrated. This method is faster than the method recently reported by Essink et al. [19] and relatively simple because complex formation is avoided. It is flexible in view of the possibility to use smaller sample volumes followed by a concentration step. Detector poisoning was not observed under the experimental conditions used. This can be explained by the fact that we used a coulometric detector instead of an amperometric detector [18].

Apomorphine is highly susceptible to autooxidation. The chemical half-life is only 39 min at conditions comparable to those in plasma in vivo . This is close to the biological half-life which was only 32 min after a single injection. This could indicate that apomorphine autooxidation may play a very significant role in its disposition, in contrast with the results mentioned in previous reports [23–27]. This could also mean that the oral route of administration is undesirable, not because of an hepatic first pass effect, but primarily because of autooxidation. New data have been produced on the stability of apomorphine and our findings throw some doubt on the data reported in the literature which were obtained without paying considerable attention to the extreme lability of the drug.

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