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Novel liquid chromatographic assay for the low-level determination of apomorphine in plasma

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Abstract

A novel HPLC assay which is rapid, reproducible and sensitive has been developed for the analysis of apomorphine in plasma. The assay incorporates boldine as an internal standard, and uses solid-phase extraction on C_{18} mini-columns for sample clean-up and concentration, so enabling quantitation of apomorphine at 500 pg/ml using fluorescence detection (λ_{ex} 270 nm, λ_{em} 450 nm). The HPLC assay comprised a 25 cm-long Techopak C_{18} column and a mobile phase of (0.25 *M* sodium dihydrogen phosphate plus 0.25% heptane sulphonic acid, to pH 3.3 with orthophosphoric acid) containing 30% (v/v) methanol and 0.003% (w/v) EDTA, run at a flow-rate of 1.5 ml/min. Calibration plots prepared in plasma were linear over the range 1–30 ng/ml, (limit of quantitation (LOQ)=490 pg/ml) with R.S.D. of 0.05% and R.E. of 5.0% at the level of 1 ng/ml. Preliminary pharmacokinetic data from two patients given apomorphine by 12 h subcutaneous infusion (patient A dose=35 mg and patient B dose=141 mg) showed apomorphine elimination from plasma to fit a two-compartment model, with initial half-lives of 8.2 and 46.6 min, elimination half-lives of 76.4 and 166.5 min and area under the plasma concentration–time curve (AUC) values of 236 and 405 ng h/ml, respectively.

Keywords: Apomorphine

1. Introduction

Apomorphine (Fig. 1) possesses dopaminergic activity, and has recently been introduced in the treatment of idiopathic Parkinson's disease, a disease caused by progressive degeneration of the central nervous system, leading to a deficiency of the neurotransmitter dopamine. Current drug therapy aims simply to correct this imbalance, with levodopa being the treatment of choice. Levodopa treatment often results in severe 'on-off' motor fluctuations which may last for 2–4 h. However, the 'off' periods can be rapidly reversed by adjunctive apomorphine treatment. Apomorphine is usually administered subcutaneously (SC), either by repeated injection or, more recently, by prolonged continuous infusion. Previous reports [1,2] have demonstrated that the time course of the effects of apomorphine, a drug with rapid onset of action (<5 min after a SC bolus), and brief duration of effect (the duration being dose-

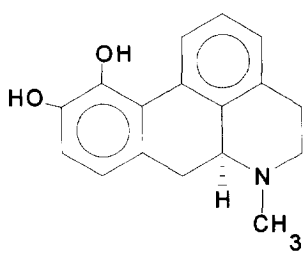

Fig. 1. Chemical structure of apomorphine.

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dependent, and lasting typically less than one hour following a bolus dose) can be predicted by its peripheral pharmacokinetics. Although SC apomorphine has been shown to appreciably reduce the number and severity of 'off' phases, periods of incapacitating immobility may still occur in some patients, despite constant rate infusion [3]. This immobility could usually be overcome by additional bolus doses of the drug. These findings, together with reports of large inter-patient variability in apomorphine pharmacokinetics by SC administration [1,2,4], indicate an obvious need for monitoring plasma drug concentrations in individual patients, with the aim of optimising clinical response and dosage schedule. To this end, a rapid and sensitive HPLC assay has been developed for the determination of apomorphine in plasma. The assay includes a solid-phase extraction step for plasma clean-up, since apomorphine was found to degrade [5] under the acidic conditions commonly used for the liquid–liquid extraction of apomorphine from plasma in previously published methods [1,2,6–8].

2. Experimental

2.1. Chemicals

Apomorphine hydrochloride was from Semat Technical (St. Albans, UK), HPLC grade methanol was from Fisons Scientific Equipment (Loughborough, UK), and all other reagents were from Sigma (Poole, UK).

2.2. Sample collection

Blood samples were collected via a venflon into cold EDTA vacutainer tubes (7 ml), transferred into polypropylene tubes containing 10 mg ascorbic acid, mixed and then stored at 4°C for a maximum of 3 h before centrifugation (1250 *g*, 10 min). The resulting plasma was immediately aspirated into polypropylene tubes and stored at −30°C until required for analysis by HPLC.

Samples were collected pre-infusion, and after the start of the infusion at times of 10, 20, 30, 45, 60 and 90 min (run-up samples), 2, 4, 8, 10 and 12 h (steady-state samples), and post-infusion at 10, 20, 30, 45, 60 and 90 min, and 2, 4, 6 and 8 h.

2.3. Solid-phase extraction procedure

Plasma samples were thawed at 4°C, centrifuged (1250 *g*, 5 min) to remove precipitated protein, and then 1–3 ml of each resulting supernatant aspirated into a polypropylene tube. Freshly prepared 2-mercaptoethanol (1%, v/v) was then added to the samples to give a final concentration of 0.01% (v/v) (i.e. 10 μl per ml sample), and the tubes placed at 4°C. Prior to extraction, boldine (the internal standard) was added to each sample to give a final concentration of 5 ng/ml [i.e. 20 μl of 250 ng/ml stock solution (in EDTA (0.1%)–ascorbic acid (0.15%)) per ml plasma], and equilibrated for 10 min at 4°C.

Varian BondElut C_{18} 1 ml/100 mg solid-phase extraction columns (Phenomenex, Macclesfield, UK) were conditioned for use by washing with 2 ml methanol followed by 2 ml water, taking care not to let the columns dry out at any stage. A 1–3 ml sample (depending on expected apomorphine concentration) was then aspirated through each column, followed by washes of 2 ml water, 1 ml 10% (v/v) methanol in water, 1 ml 20% (v/v) methanol in water and 1 ml 50% (v/v) methanol in water, again taking care not to let the columns dry out at any stage. Apomorphine and boldine were then eluted using 2×200 μl 0.1 *M* methanolic HCl into 1 ml volumetric flasks containing 400 μl sodium metabisulphite (1%, w/v), and made up to volume using a solution of EDTA (0.1%, w/v) and ascorbic acid (0.15%, w/v) for analysis by HPLC.

2.4. Liquid chromatography

The HPLC system consisted of a Jasko pump (PU-920), autosampler (AS-950) and fluorescence detector (FP-920, fitted with an R3788-01 photomultiplier tube) (Jasko, Great Dunmow, UK), and a CI4000 integrator from Thermo Separations Products (Stone Staffs, UK). The HPLC column was a 10 μm Techopak C_{18}, 250×4 mm I.D. column fitted with a 2 cm-long guard column of the same packing material (HPLC Technology, Macclesfield, UK). The mobile phase was (0.25 *M* sodium dihydrogen phosphate plus 0.25% heptane sulphonic acid, to pH 3.3 with orthophosphoric acid) containing 30% (v/v) methanol and 0.003% (w/v) EDTA, run at a flow-rate of 1.5 ml/min. Detection was at an excitation

wavelength of 270 nm and an emission wavelength of 450 nm (gain 1000, attenuation 1). The injection volume was 50 μl.

2.5. Calibration plot

Calibration plots were prepared using pooled plasma on two separate occasions, with the ranges 1–10 ng/ml (n=5) and 1–30 ng/ml (n=6). A representative chromatogram of apomorphine (1 ng/ml) and boldine (5 ng/ml) extracted from spiked plasma is shown in Fig. 2. Linearity was assessed using the correlation coefficient r and the relative errors of all points.

2.6. Accuracy and precision

Precision and accuracy of apomorphine determination in plasma at 0.5, 1, 10 and 30 ng/ml were determined. Between-run precision and accuracy were also determined using the calibration standards prepared for each assay.

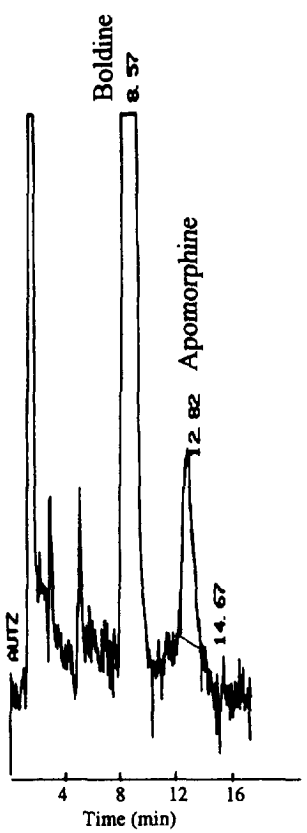

Fig. 2. Representative chromatogram of apomorphine (1 ng/ml) and boldine (5 ng/ml) extracted from 3 ml spiked plasma.

2.7. Stability indication of HPLC assay

Alignots (1 ml) of apomorphine hydrochloride (2.5 mg/ml) were heated with either (a) 1 ml 1 *M* NaOH, (b) 1 ml 1 *M* HCl, (c) 1 ml 20 vol H_2O_2 or (d) 1 ml H_2O, for 2 h in the dark at 85°C. The solutions were then cooled, and to solutions (a) and (b) were added 1 ml of 1 *M* HCl and 1 ml of 1 *M* NaOH, respectively. Solutions were then diluted to 25 ng/ml for assay, and their apomorphine content compared to that of a control stored at 4°C.

2.8. Stability in plasma

The stability of apomorphine and boldine in plasma during the storage steps of the assay procedure (10 min, 4°C and 1–2 min, 24°C) were investigated as follows: 6 ml volumes of plasma were spiked with 2-mercaptoethanol (to 0.01%, v/v), apomorphine (to 10 ng/ml) and boldine (to 5 ng/ml), and incubated at either 4°C or 24°C for 60 min. Samples (1 ml) were taken for analysis at times of 0, 10, 20, 30, 45 and 60 min incubation at each temperature. As it has previously been reported [9] that apomorphine is stable for 70 days in frozen plasma containing 5 m*M* ascorbate, it was not considered necessary to repeat this investigation.

2.9. Stability in SPE elution solvent

A 3-ml volume of a solution of apomorphine (1 ng/ml) and boldine (5 ng/ml) [in EDTA (0.1%, w/v) and ascorbic acid (0.15%, w/v)] were extracted using an SPE column, as described above, and the resulting extract placed in an autosampler at 4°C. The extract was analysed at times: 0, 20, 40, 60 and 120 min post-extraction, to determine whether any degradation of apomorphine or boldine was occurring in the highly acidic elution solvent.

3. Results and discussion

Preliminary method development studies on apomorphine revealed a number of characteristics

which were found to be important in the analysis of patient samples for pharmacokinetic studies. Apomorphine was found to bind to glass vials, the solid-phase extraction manifold, the HPLC column and the autosampler, particularly when used at high concentration (10 μg/ml), necessitating the use of silanised glassware or polypropylene tubes for the handling of all solutions containing this drug. The HPLC column, autosampler and SPE manifold were cleaned using washes of methanol followed by propanol, and to prevent carryover, the autosampler rinse solvent was 50% methanol in water. Carryover was prevented on the HPLC column by ensuring that no injection of >100 ng/ml was injected and the column was cleaned (as above) after every 30–40 injections. Apomorphine was also found to react with the commonly-used antioxidant sodium metabisulphite, resulting in the formation of an adduct [5]. Sodium metabisulphite gave the best protection from degradation during extraction and elution however, but adduct formation could be prevented by the addition of EDTA (0.1%, w/v)–ascorbic acid (0.15%, w/v) solution. The LOQ of the assay was 490 pg/ml. However, the up to five-fold sample concentration effect (1–5 ml of sample may be extracted) can increase the achievable limit of detection of the method. This LOQ compares favourably to previously reported assay methods which use solvent extraction and electrochemical detection (LOQs of 0.1 [2], 0.4 [1,10–12] and 0.8 ng/ml [13]) and by using solid-phase extraction for sample clean-up is rapid, reproducible and easily automated.

3.1. Calibration plot

Calibration plots prepared using spiked plasma were linear over the range of: (a) 1–10 ng/ml, with a regression equation of $y=0.088x-0.022$, $r=0.99$, $n=5$, and (b) 1–30 ng/ml, with a regression equation of $y=0.083x+0.032$, $r=0.99$, $n=6$, (performed on two separate occasions, where x=apomorphine concentration and y=the apomorphine to boldine peak-height ratio). The concentration of apomorphine in plasma was calculated using the (unweighted) linear regression equation appropriate for the apomorphine concentration, and verified by comparison with an extracted spiked plasma standard of a similar concentration. The LOQ of apomorphine in plasma was calculated using an extrapolation of the point $2Sy/x$ to the x-axis of the calibration plot, where Sy/x is given by:

$$Sy/x = \sqrt{\frac{\sum (yi' - yi)^2}{n-2}}$$

Table 1 shows the calibration data together with the quality control data: the theoretical concentrations of apomorphine in spiked plasma, the corresponding observed concentrations determined from the calibration plot, the corresponding R.S.D. values for the observed concentrations and the relative errors (R.E.).

Absolute recovery of apomorphine and boldine extracted from plasma was 76.0 and 77.8%, respectively.

3.2. Accuracy and precision

Accuracy and precision of apomorphine extraction from 1 to 3 ml spiked plasma at 0.5, 1, 10 and 30 ng/ml are presented in Table 1.

3.3. Stability indication of HPLC assay

Degradation studies on apomorphine at elevated temperature revealed a complete loss of analyte under the basic conditions studied, incubation with HCl gave an 0.7% loss, incubation with H_2O_2 gave an 78.2% loss and incubation with water gave a 24.0% loss. Dilution of the NaOH-degraded solution to 2.5 μg/ml revealed the presence of apomorphine and three degradation products, of relative retention times (t_{RR}) to apomorphine of 0.67, 0.75 and 1.5. Dilution of the HCl-degraded solution to 2.5 μg/ml showed the presence of a degradation peak of t_{RR} 0.81, and dilution of the H_2O_2-degraded solution to 2.5 μg/ml did not reveal any degradation products. Boldine elutes at t_{RR} 0.65, and so would only mask the presence of one degradation product, formed under basic conditions (not present in this assay). The degradation of boldine under the same conditions gave a 3.2% loss in H_2O, a 6.4% loss in H_2O_2, an 0.7% loss under acidic conditions and an 88.8% loss under basic conditions. No boldine

Table 1
Accuracy and precision of the method

C^a (ng/ml)	Volume of plasma extracted (ml)	Mean apomorphine /boldine peak height ratio	C^b (ng/ml)	Apomorphine assay (% of theoretical)	R.S.D. (%)	R.E.[c] (%)	Within-run precision C.V. (%)	Between-run precision C.V. (%)
Calibration standards								
1	3	0.131						
2.5	3	0.231						
5	1	0.447						
10	1	0.901						
15	1	1.331						
30	1	2.538						
Calibration quality controls								
0.5	3	0.025	0.53	106.0	0.04	6.00		
1	3	0.070	1.05	105.0	0.05	5.00	5.20 (n=5)	6.41 (n=5)
10	1	0.860	10.02	100.2	1.04	0.02	2.69 (n=5)	5.07 (n=5)
30	1	2.549	30.33	101.1	0.87	1.10	3.38 (n=3)	–

[a]Theoretical concentrations of standard solutions of apomorphine in plasma.
[b]Mean concentration of apomorphine calculated for standard solutions in plasma from the equations: $y=0.088x-0.022$ (1–10 ng/ml) and $y=0.083x+0.032$ (10–30 ng/ml).
[c] Relative error.

degradation products were observed. The assay was therefore considered to be stability-indicating.

3.4. Stability in plasma

Apomorphine and boldine were found to be stable in plasma plus 2-mercaptoethanol, both at 4°C and 24°C, as shown in Table 2. The peak heights and peak-height ratios remained constant over the time period investigated, and no degradation peaks were observed on the chromatograms, indicating that apomorphine and boldine were stable in plasma for at least 45 min (exceeding the assay storage time of 10 min) at both room temperature and 4°C.

Table 2
Stability of apomorphine and boldine in plasma plus 2-mercaptoethanol (0.01%, v/v)

Incubation time (min)	Apomorphine/boldine peak-height ratio	Apomorphine concentration (ng/ml)	Apomorphine remaining (%)
4°C			
0	0.843	9.8	
10	0.883	10.3	
20	0.870	10.1	
30	0.880	10.2	
45	0.835	9.7	99.0
60	–	–	–
24°C			
0	0.829	9.6	
10	0.837	9.7	
20	0.887	10.3	
30	0.853	9.9	
45	0.837	9.7	101.0
60	0.854	9.9	103.1

3.5. Stability in SPE elution solvent

A 3-ml volume of apomorphine–boldine solution was extracted and analysed as described above. The apomorphine–boldine peak-height ratios after 0, 20, 40, 60 and 120 min were as follows: 0.0566, 0.0586, 0.0563, 0.0590 and 0.0568 (C.V.=2.16%). The peak heights and peak-height ratios remained constant over the time period investigated, and no degradation peaks were observed on the chromatograms, indicating that the extracts were stable for at least 120 min at 4°C.

3.6. Pharmacokinetic studies

Plasma concentration–time data from two patients (A and B) were collected, and are presented in Fig. 3 (whole infusion course) and Fig. 4 (post-infusion data for patient B). It was possible to quantitate plasma concentrations of less than the LOQ of 0.5 ng/ml (3 ml plasma extracted) by extracting 5-ml volumes of low level samples. Steady-state plasma concentrations (CPSS), for comparative purposes, were calculated using the plasma concentrations determined at times from 2 to 12 h of the infusion. Areas under the plasma concentration–time curve (AUCs) were calculated using the trapezoidal rule

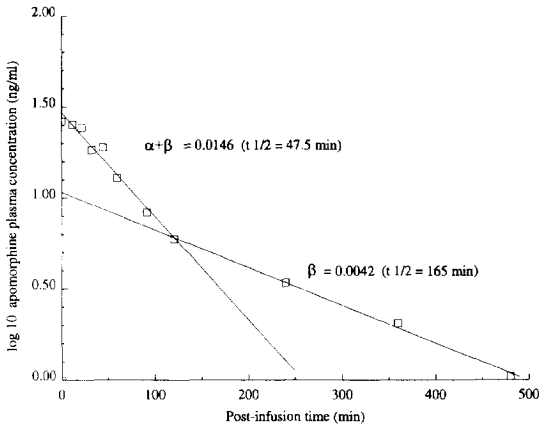

Fig. 4. Semi-logarithmic plot of the post-infusion plasma concentrations of apomorphine given by subcutaneous infusion (141 mg/18 ml over 12 h).

(0–12 h time points) plus the AUC of a computer-simulated elimination curve (as described below). Cl/F data were calculated using dose/AUC. Plasma half-lives were calculated from patient data using a computer modelling program (Statis3, Clydesoft, Larkhall, UK) which showed a two-compartmental model of elimination to best fit the data. These pharmacokinetic data are presented in Table 3, and are compared with previously published results [1,2]. It can be seen from Table 3 that the data obtained in

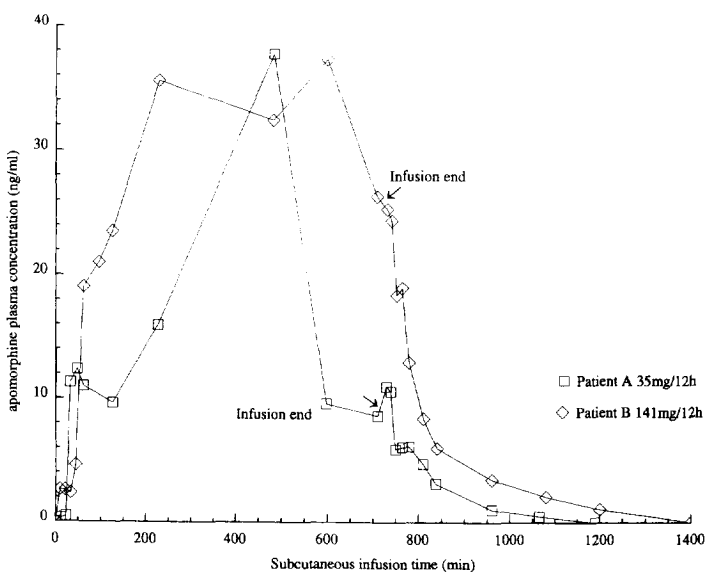

Fig. 3. Plasma concentrations of apomorphine in two patients given apomorphine by 12-h subcutaneous infusion.

Table 3
Pharmacokinetic parameters calculated for apomorphine by subcutaneous 12 h infusion

Patient or study	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (min)	Cl/F (ml/min)	$AUC_{(0-\infty)}$ (ng h/ml)	Dose	Flow-rate (mg/h)	CPSS (ng/ml)	Last time point (min)
A	8.2	76.4	2471	236.1	35 mg/12 h	2.91	16.3	480
B	46.6	166.5	5800	405.2	141 mg/12 h	11.75	31.0	480
Nicolle et al. [2]	17.6±1.5	835±973 (228[a])	[4000±1900]	–	[84 mg/24 h] (n=5)	[5.4] (n=5)	27.9±9.3 (n=5)	240
Gancher et al. [1]	4.8±1.1	33.6±3.9	–	–	[36 mg/6 h] (n=1[b])	[6.0] (n=1[b])	31.7[b] (n=1[b])	120

Figures given in square brackets have been estimated from data given in units of mg/kg, using 60 kg as an estimated patient weight.
[a]Represents the median value.
[b]Represents data taken from a graph presented in Ref. [1].

our study are comparable to those previously published. It is also apparent that the distribution and elimination half-lives determined for the two patients are significantly different. This raises questions concerning the influence of the concentration and rate of infusion on the pharmacokinetic parameters of apomorphine. In this study, patient A received 35 mg apomorphine in 18 ml over 12 h (i.e. 2.91 mg/h), whereas patient B received 141 mg apomorphine in 18 ml over 12 h (i.e. 11.75 mg/h). The AUC value calculated for patient B was considerably greater than for patient A, as expected. A large inter-patient variability in apomorphine pharmacokinetics has previously been reported (ten-fold difference in C_{max} and five-fold difference in AUC for a 30 μg/kg dose administered by SC bolus to 12 patients [1]) which may explain the differences in half-lives calculated, although further studies on the effect of dose and concentration of infusions are indicated before a pharmacokinetic/pharmacodynamic study is performed. Fig. 3 shows a 'high' point in the steady-state apomorphine plasma concentrations of patient A at 8 h. This may have resulted from a pressure being applied to the SC infusion site, or a malfunction in the infusion device. Such data points cannot be excluded, as they represent the true plasma concentrations experienced, but highlight the difficulty in obtaining good quality pharmacokinetic data from human subjects, particularly when the SC rather than the IV route is used.

In conclusion, this method has been shown to be sensitive enough for use in pharmacokinetic studies on patients receiving low dose apomorphine by continuous SC infusion.

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