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DETERMINATION OF CLONIDINE IN HUMAN PLASMA BY COLD ON-COLUMN INJECTION CAPILLARY GAS CHROMATOGRAPHY–SELECTED-ION MONITORING–MASS SPECTROMETRY

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SUMMARY

Clonidine [Catapres™, 2-(2,6-dichlorophenylimino)-2-imidazoline hydrochloride], a potent, antihypertensive drug, given in a dosage of 0.1–0.3 mg, yields plasma levels in the ng/ml to pg/ml range. A method for the determination of clonidine from human plasma was developed, which includes a simple, one-step solvent extraction followed by a one-step derivatization with N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide to form a *tert.*-butyldimethylsilyl (TBDMS) derivative. Levels of clonidine (TBDMS) were determined by cold on-column injection capillary gas chromatography–selected-ion monitoring–mass spectrometry. Precision of the method at the 1 ng/ml level was ±2.4%.

INTRODUCTION

Clonidine [Catapres™, 2-(2,6-dichlorophenylimino)-2-imidazoline hydrochloride] has a unique history. Originally investigated as a nasal decongestant, this potent, lipophilic agent was among the first drugs to be clearly classified as centrally acting [1–3]. Studies indicate that clonidine preferentially stimulates the α_2-adrenoreceptors in the area of the pons and medulla of the brain stem, resulting in a reduction of sympathetic tone and lowered arterial blood pressure. Clonidine has been used effectively in the treatment of mild to severe hypertension and has been used successfully in combination with other antihypertensive agents. Clonidine, given in oral doses of 0.1–0.3 mg twice daily, yields peak plasma

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levels in the ng/ml to pg/ml range [1–3]. Clonidine, like other centrally acting antihypertensives, elicits a number of side-effects [1,3]. Some of these side-effects have recently been used therapeutically [4–10], therefore, the determination of clonidine may become desirable in other clinical settings.

The low therapeutic plasma levels of this potent antihypertensive drug (pg/ml–ng/ml) make its determination a challenge for the analyst. Several methods have been devised for the determination of clonidine and the most sensitive of these are the radioimmunoassay (RIA) method of Arndts et al. [11] and the gas chromatographic (GC)–electron-capture negative-ion chemical-ionization mass spectrometric (MS) method of Murray and Davies [12]. Both of these techniques register a minimum level for clonidine determination in human plasma of 10 pg/ml. However, therapeutic doses of clonidine yield plasma levels of the order of 200–1000 pg/ml. Obviously, either of these methods would provide excellent data at those levels. On the other hand, most analytical chemists are unfamiliar and/or ill equipped to raise antisera in rabbits and other manipulations required by RIA techniques, few analysts have access to a mass spectrometer with electron-capture negative-ion chemical-ionization capability, and fewer still have the expertise to successfully operate such an MS system. The methods which use GC generally suffer from multi-step extractions and/or column liquid chromatographic separations, multi-step derivatization procedures, and require 4–5 ml of plasma [13–17].

It was our desire to develop a simple, rapid capillary GC method using cold on-column injection and selected-ion monitoring (SIM) MS detection for the determination of clonidine in human plasma at therapeutic levels. We also wanted to use methodology that could be readily transferred to the newly developed mass-selective–ion trap GC–MS detector system [18,19]. Compatibility with these new GC–MS detector systems was important as they have been optimized for capillary GC–MS applications, provide SIM–MS selectivity and sensitivity at a fraction of the cost of larger MS systems, and require less operator training and experience.

EXPERIMENTAL

Materials

Clonidine for use as a standard was kindly provided by C.H. Boehringer Sohn (Ingelheim, F.R.G.). The internal standard (I.S.), benzimidazole, was obtained from Chem Service (West Chester, PA, U.S.A.); ammonium hydroxide, 29.3% NH_3 in water, and 1-butanol, reagent grade, from Mallinckrodt (Paris, KY, U.S.A.); methanol, hexane, and cyclohexane, distilled-in-glass grade, from Burdick and Jackson Labs. (Richmond, CA, U.S.A.); trimethylchlorosilane (TMCS) and N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) from Pierce (Rockford, IL, U.S.A.); and 13×100 mm and 16×150 mm Pyrex screw-cap culture tubes from Corning (Corning, NY, U.S.A.). All reagents were used as received.

A flow chart of our analytical procedure for the determination of clonidine in human plasma is given in Fig. 1.

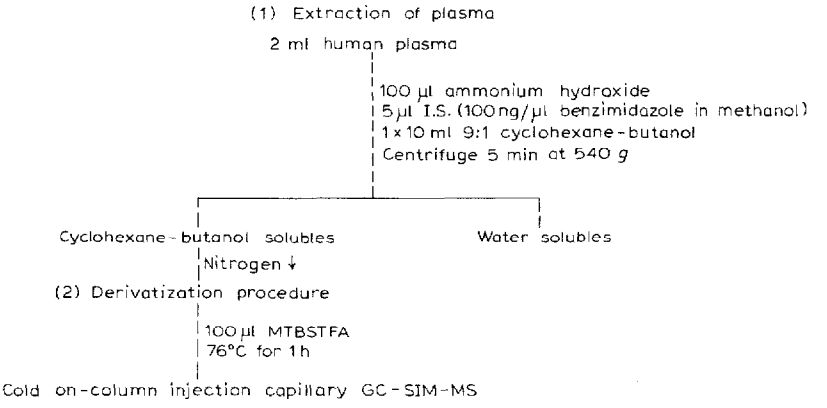

Fig. 1. Analytical procedure for the determination of clonidine in human plasma.

Extraction of plasma

Clean 16×150 mm Pyrex screw-cap culture tubes with PTFE cap liners were silanized with TMCS before use by rinsing them first with methanol, followed by a 5% solution of TMCS in hexane, and twice more with methanol. The silanized tubes were allowed to air-dry at room temperature.

Human plasma (2 ml) was transferred to a silanized 16×150 mm culture tube. To the plasma in the tube were added 100 µl of ammonium hydroxide (29.3% NH_3), 5 µl of I.S. solution (100 ng/µl benzimidazole in methanol), and 10 ml of 9:1 cyclohexane–butanol [13]. The solution was thoroughly mixed, then vortexed for 30 s on a Lab-Line Super-Mixer, and centrifuged for 5 min at 540 *g*. The organic layer (cyclohexane–butanol solubles) was transferred to a second 16×150 mm screw-cap culture tube. All but about 2 ml of the solvent was evaporated with a stream of dry nitrogen under mild heat (45°C) and the remaining fraction was transferred to a 13×100 mm Pyrex screw-cap culture tube with PTFE cap liner which had been tapered in a flame and silanized as above.

Derivatization procedure

The remaining solvent was evaporated from the extract in a stream of dry nitrogen with mild heating (45°C), 100 µl of MTBSTFA were added, and the tapered culture tube was capped and placed in a heating block at 76°C for 1 h.

Cold on-column injection capillary GC

The capillary GC separation of the derivatized plasma extract was performed with a Hewlett-Packard 5840 GC system, which is an integral part of the Hewlett-Packard 5985B GC–MS system, equipped with a laboratory-constructed cold on-column inlet system [20]. The fused-silica immobilized stationary phase SE-54 capillary columns used in this work had stationary phase films that were about 0.05 µm thick and were prepared by the method of Arrendale and Martin [21]. Capillary GC conditions were as follows: column dimensions, 30 m×0.3 mm I.D.; temperature program, 100°C for 1 min, 100–300°C at 6°C/min; column flow-rate (helium), 40 cm/s; injection volume, 1 µl MTBSTFA. Chromatograms

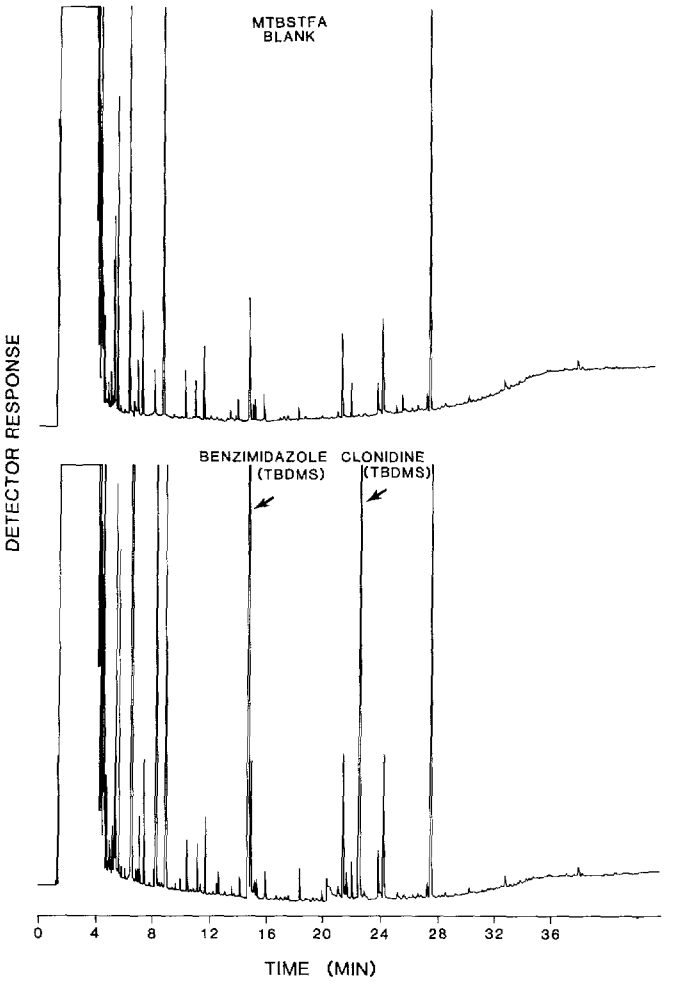

Fig. 2. Cold on-column injection capillary GC–flame ionization detection separation of MTBSTFA solvent blank (upper chromatogram) and standard benzimidazole (I.S.) and clonidine TBDMS derivatives (lower chromatogram).

showing the cold on-column injection capillary GC separation (flame ionization detector) of the MTBSTFA blank and benzimidazole and clonidine (*tert.*-butyldimethylsilyl, TBDMS) standards are given in Fig. 2.

Capillary GC–SIM–MS

The mass spectrometer used in this work was a Hewlett-Packard 5985B GC–MS system. The fused-silica SE-54 capillary column was interfaced to the MS source through a piece of 0.15 mm I.D. vitreous silica tubing which had been deactivated with OV-1701-vinyl [21,22]. The connection between the 0.3 mm I.D. fused-silica SE-54 column and the 0.15 mm I.D. vitreous silica interface tubing was made with a laboratory-constructed open split interface or a Supelco capillary butt connector and a double-tapered Vespel ferrule [22]. In either case,

the entrance end of the 0.15 mm I.D. interface tube was inserted a few millimeters inside the exit end of the 0.3 mm I.D. SE-54 capillary column. The SIM program of the Hewlett-Packard 5985B using both an internal spiking technique with benzimidazole as the I.S. and an external standard method was used for the determination of clonidine (TBDMS) in human plasma. Analyses with SIM involve calibration of the MS system to correct for mass defect or calibration inaccuracies which assures that the mass chosen is at the top of the MS ion peak for best sensitivity and selectivity [16–19]. The 70-eV electron-impact MS analyses (Fig. 3) of the TBDMS derivatives of benzimidazole (I.S.) and clonidine showed that the benzimidazole spectrum contains a molecular ion at m/z 232 and a fragment ion at m/z 175 resulting from the loss of the *tert.*-butyl group, and the clonidine spectrum had a weak molecular ion at m/z 343, another weak ion at m/z 286 from the loss of the *tert.*-butyl group, and a very intense ion at m/z 252. The molecular ion (m/z 232) was chosen for SIM–MS of benzimidazole (I.S.) and the intense ion at m/z 252 was chosen for quantitative determination of clonidine. A set of typical SIM–MS run parameters are given in Table I.

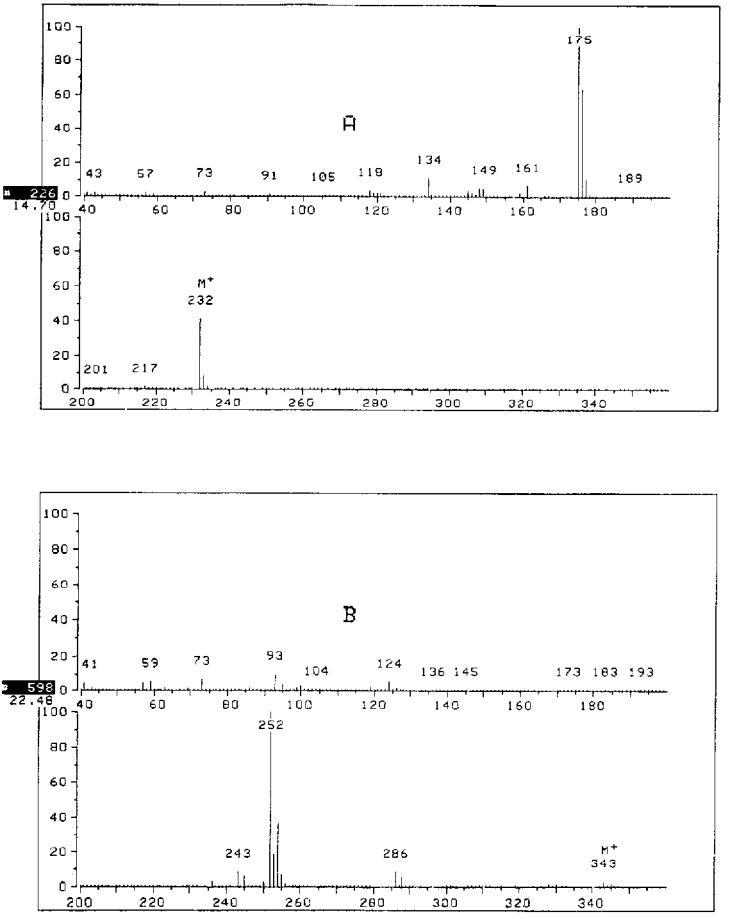

Fig. 3. Electron-impact (70 eV) mass spectra of (A) benzimidazole (I.S.) and (B) clonidine TBDMS derivatives.

TABLE I

GC-SIM-MS RUN PARAMETERS

Parameter	Benzimidazole (TBDMS)	Clonidine (TBDMS)
Start time (min)	10	16
Run time (min)	6	6
Mass/dwell*	232.1/500	252.1/500

*Dwell = time in ms.

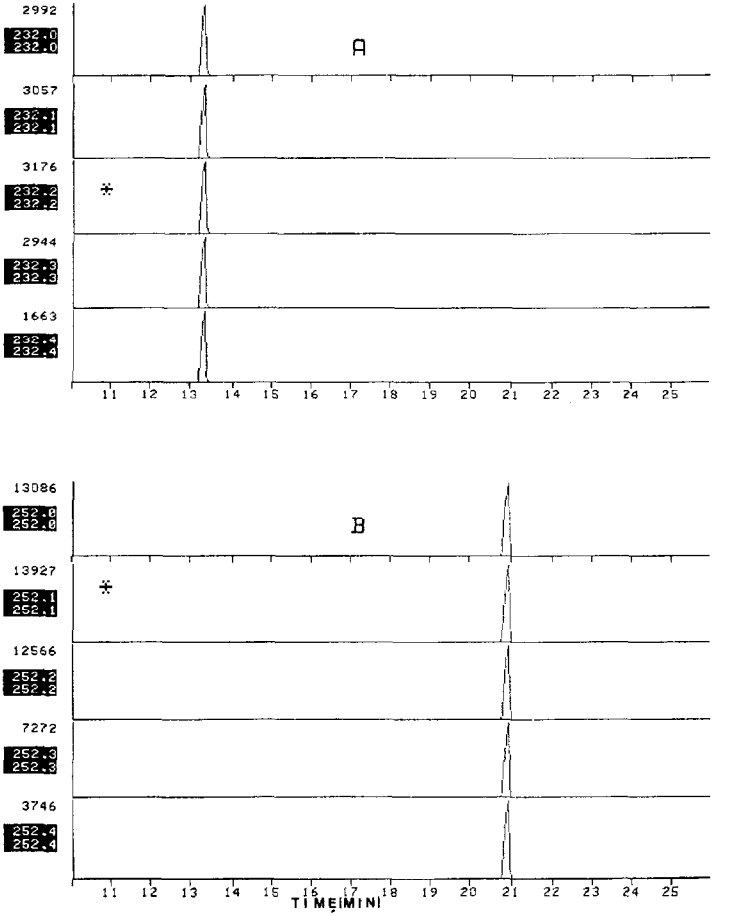

Fig. 4. Chromatogram of the cold on-column capillary GC-SIM-MS analyses of standard (A) benzimidazole, m/z 232 (I.S.), and (B) clonidine, m/z 252, TBDMS derivative for the determination of the highest abundance ions for use in GC-SIM-MS analyses of clonidine in human plasma (abundances are printed above each ion selected on the left margin of the GC-SIM-MS chromatogram).

The mass within 0.1 a.m.u. for SIM, i.e. the top of each MS peak, was chosen by running a standard mixture of clonidine and benzimidazole (TBDMS), monitoring ions near the even mass number (232 for benzimidazole and 252 for clon-

idine), and selecting the most intense ion in each instance as measured by its peak area. An example of this determination, shown in Fig. 4, indicates the most intense ion for each compound with an asterisk.

RESULTS AND DISCUSSION

Investigations of the plasma levels of a drug after oral or intravenous dosage involves sampling at time 0 (before dosage) and at fixed intervals thereafter. Our goals in the development of this methodology included reduction in the amount of plasma needed per analysis and the simplification of extraction and derivatization procedures. The cold on-column injection technique was chosen for our capillary GC because it provides several advantages over conventional vaporizing injection modes such as (a) elimination of thermal or catalytic decomposition due to rapid heating or contact with hot inlet parts, (b) elimination of discrimination between low- and high-boiling components resulting in excellent reproducibility and linearity of response, and (c) highest capillary GC sensitivity as 1-μl volumes of liquid samples were injected directly onto the capillary column through an inlet maintained at a temperature below the boiling point of the solvent [23–31]. Operation of a modern, computer-controlled GC–MS system in the SIM mode provides both sensitivity and selectivity [32–35]. By choosing ions that are characteristic of the compounds of interest, the mass spectrometer in the SIM mode functions as a selective detector. The mass spectrometer, when adjusted to selected masses for defined periods of time, provides detection limits that are 2 or 3 orders of magnitude lower than are possible in the scanning mode.

Silylation of a compound generally improves its GC–MS response as measured by an increase in total ion current obtained per amount of compound injected. Often this results from the production of relatively few high-intensity ions upon electron bombardment and to some degree from improved GC peak shape. Reaction of clonidine with MTBSTFA to form its TBDMS derivative improved both its GC–MS response and chromatographic elution characteristics. Another important function of silylation was the conversion of many polar, non-volatile contaminants in the organic plasma extract into GC volatile compounds that could be eluted from the capillary GC column without interfering in the determination of clonidine (TBDMS).

The efficiency of extraction of clonidine from human plasma was determined by exhaustive extraction of plasma spiked with standard clonidine. Recovery was quantitative, at the 1 ng/ml level, in the first partitioning step with 9:1 cyclohexane–butanol as no clonidine was detected in subsequent partitioning steps. The reproducibility of extraction of clonidine from spiked human plasma was also very good as discussed below. It should be noted here that standard runs using this methodology should be made with fresh human plasma or fresh plasma that has been frozen. Serological standards of human plasma which must be reconstituted with water or human plasma that has aged for long periods of time at room temperature are unsuitable as they contain breakdown products which interfere with the chromatographic process in cold on-column injection capillary GC. However, any human plasma may be used as a standard for others as

reproducibility between different human plasma samples was excellent. The analyses of two different plasma samples spiked with 1 ng/ml clonidine are shown in Fig. 5.

The formation of TBDMS derivatives of clonidine and benzimidazole (I.S.) were complete as reactions of large amounts of these compounds gave single peaks by capillary GC and GC–MS analyses. Formation of trimethylsilyl (TMS) derivatives of these compounds using N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was also successful, but the intensity of the ion in SIM–MS analogous to the m/z 252 ion used for determination of clonidine (TBDMS) was ten times less, therefore, the TBDMS derivatives were chosen over the TMS derivatives. The TBDMS derivatives of clonidine and benzimidazole are stable in pure form over long periods of time, but are less stable in the derivatized plasma extracts and, as with all silyl derivatives used for quantitative determinations, should be analyzed soon after derivative formation.

Cold on-column injection is the most reproducible, sensitive, and quantitative technique for capillary GC [23–31]. Determination of clonidine by cold on-column injection capillary GC–SIM–MS reflects this capability. Table II gives data for the determination of clonidine in three different plasma samples (spiked with 1 ng/ml clonidine) using the I.S. method. Each plasma extract was analyzed three times and the relative error for the nine runs was 2.1%. It should be emphasized here that these were manual injections using a laboratory-constructed cold on-column inlet and more modern, sophisticated cold on-column inlets equipped with autoinjection systems would be expected to yield even better data. Determination of clonidine using an external standard method at the 1 ng/ml clonidine level taking care to reproduce injection volumes as closely as possible by manual injection yielded a relative error of only 3.2%. Again more modern equipment would be expected to yield even better results. Response curves in the therapeutic range (200–1000 pg/ml) were linear and passed through the origin.

Comparison of results after connection of the capillary column to the MS source with the open split interface or with a butt connector showed that the latter gave

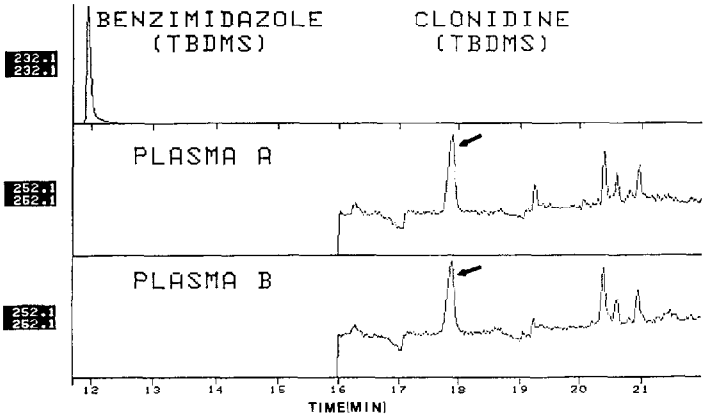

Fig. 5. Chromatogram of the cold on-column injection capillary GC–SIM–MS analyses of two different plasma samples, A and B, which were each spiked with 1 ng/ml standard clonidine.

TABLE II

REPRODUCIBILITY OF I.S. METHOD FOR CLONIDINE DETERMINATION

Plasma sample*	Run No.	Response factor (K)**
A	1	2.79
A	2	2.88
A	3	2.74
B	1	2.75
B	2	2.79
B	3	2.89
C	1	2.73
C	2	2.77
C	3	2.82
Mean ± S.D. (n=9)		2.79 ± 0.07
Error		2.4%

*Human plasma spiked with 1 ng/ml clonidine.
**K = response factor = (area x/amount x)/(area I.S./amount I.S.).

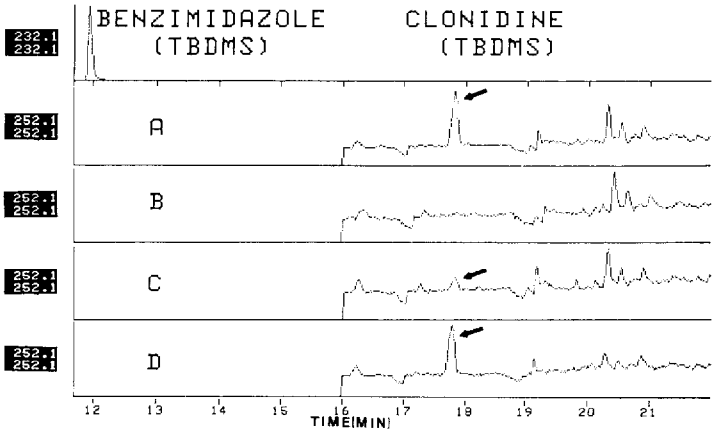

Fig. 6. Determination of clonidine in the plasma of a patient after a 100-μg oral dose: (A) time 0, plasma taken prior to dosage spiked with 1 ng/ml standard clonidine; (B) time 0, plasma taken prior to dosage; (C) plasma taken 15 min after oral dosage containing 0.177 ng/ml clonodine; (D) plasma taken 30 min after oral dosage containing 0.904 ng/ml clonidine.

slightly better sensitivity and was thus used for most determinations. Obviously, the sensitivity of this methodology also depends greatly upon the general condition of the MS source and the MS electron multiplier. Analyses after installation of a newly cleaned MS source and a new electron multiplier yielded even better sensitivity than the 200 pg/ml level. Determination of clonidine in the plasma of a patient after a 100-μg oral dose is illustrated in Fig. 6. The response of benzimidazole (I.S.) is shown at the top, followed by that for plasma prior to dosage (time 0) spiked with 1 ng/ml clonidine; time 0 plasma; plasma levels of clonidine 15 min after oral dosage (0.177 ng/ml); and after 30 min (0.904 ng/ml). A graph

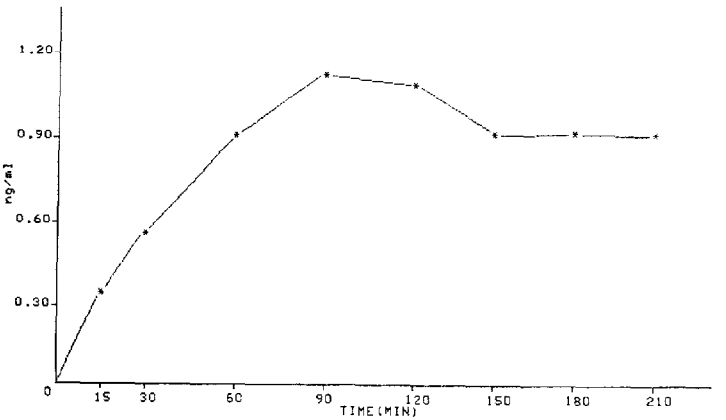

Fig. 7. Levels of clonidine in the plasma of a patient given a single 100-μg oral dose.

depicting the levels of clonidine over a 3.5-h period in the plasma of a patient given an oral dose of clonidine (100 μg) is shown in Fig. 7.

This method demonstrates the applicability of the cold on-column injection technique for the capillary GC analyses of trace components in human plasma. Other GC methods for the determination of clonidine or other drugs in plasma could significantly improve reproducibility and/or increase sensitivity by application of cold on-column capillary GC instead of packed column GC or capillary GC with split/splitless injection [12,17]. The determination of clonidine in human plasma at therapeutic levels was greatly simplified by employing a single, one-step extraction of plasma and a one-step derivatization procedure to yield TBDMS derivatives of clonidine and benzimidazole (I.S.). These silyl derivatives exhibited good chromatographic characteristics using cold on-column injection capillary GC and yielded intense ions which were detected with selectivity and sensitivity by SIM–MS.

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