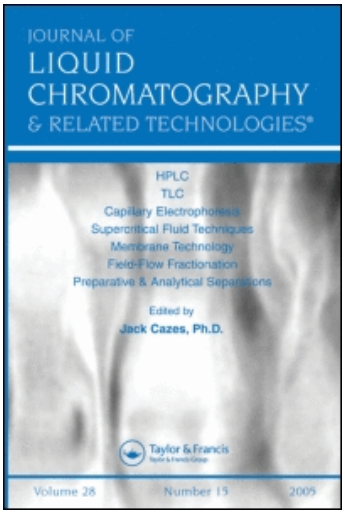


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# AN IMPROVED METHOD FOR THE DETERMINATION OF RESERPINE IN PLASMA USING LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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A liquid chromatographic method coupled with fluorescence detection was developed to measure plasma reserpine concentrations. After extraction from 3 ml of plasma, the reserpine and internal standard (methyl-18-triethoxy benzoyl reserpate) residues were oxidized to their respective fluorophors by vanadium pentoxide and chromatographed on a reversed phase trimethylsilyl column. These compounds were detected at excitation wave length 460 nm and analyzed at 570 nm. The minimum quantifiable level was ca 0.3 ng/ml and the absolute recovery was determined to be between 78-83%. The coefficient of variation was less than 9% for day-to-day and within run analyses. This method is suitable for pharmacokinetic studies of reserpine in man.

## INTRODUCTION

Until recently, the measurement of reserpine and its metabolites in plasma has been difficult because of the unavailability of sufficiently sensitive and specific methods of



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analysis. A review of the analytical methods for reserpine has been reported (1). In terms of specificity and sensitivity, two methods appear to be most useful for determining reserpine in plasma, especially in pharmacokinetic studies. Tripp et al. (2) developed a very sensitive procedure for reserpine in plasma based upon thin-layer chromatography followed by development of a fluorophor with acetic acid and scanned with a densitometer. Errors were reduced by the addition of an internal standard. The known metabolites of reserpine did not interfere. A disadvantage of this method is the lengthy development time (50 mins.), exposure to acetic acid vapors (1.5 hours) and total development time of the fluorophor (18-24 hours). In another method, Sam (3) used high performance liquid chromatography with fluorescence detection to achieve a sensitive procedure for reserpine in equine plasma. This method employs the precolumn derivitization of reserpine into a highly fluorescent oxidized derivative developed by Urbanyi and Stover (4). Although this method is sensitive and rapid, it lacked an internal standard protocol which would eliminate quantitative extraction and volume correction factors, and perhaps improved overall precision.

Our laboratory has recently been involved in the analysis of reserpine in plasma during a specific time period of its pharmacokinetic profile as part of a neuroendocrine study.

In 1981 one of us (GMA) reported that 0.5 mg reserpine given intramuscularly caused a marked elevation of plasma prolactin in

normal human male volunteers. This elevation was significantly larger than when the same group was challenged with 0.5 mg haloperidol (a potent neuroleptic drug) (5). A similar observation had been made in chronic schizophrenic patients by Meltzer (6). Further work at this institute has demonstrated that the prolactin response to 0.5 mg reserpine is markedly different in normal volunteers when compared to matched drug free schizophrenics (7). Clearly of interest is whether this response difference is due to a pharmacokinetic or pharmacodynamic effect. This prompted us to combine the merits of the above two methods i.e. using a liquid chromatographic method with a rapid pre-column derivitization reaction and the use of an internal standard that is chemically related to reserpine into a simple rapid, sensitive and specific procedure which can be used to process a large number of plasma samples on a daily basis. Although the method was developed specifically for this purpose it has much wider application in neuroendocrinology and neuropharmacology.



## MATERIALS

### Reagents:

Acetonitrile (UV grade) and methyl-tert-butyl ether were obtained from Burdick and Jackson Laboratories (Muskegon, MI). n-Heptane (spectraanalyzed grade) methanol ("HPLC" grade) were obtained from Fisher Scientific Co. (Fairlawn, NJ). Isoamyl alcohol (Sigma Chemical Co., St. Louis, MO), sodium heptane sulfonate

(Eastman Kodak Co., Rochester, NY) and triethylamine (Aldrich Chemical Co., Milwaukee, WI) were used without further purification. Reagent grade acetic acid, sodium acetate, phosphoric acid, vanadium pentoxide were obtained from Fisher Scientific Co. (Fairlawn, NJ). Distilled water was passed through a water purification system before use (Milli-Q, Millipore Corp., Bedford, MA).

Reserpine, methyl-18-triethoxybenzoylreserpate, reserpic acid, syringomethylreserpate and methylreserpate were all kindly supplied by Dr. S.L. Tripp, Ciba-Geigy Corp. (Ardsley, NY).

Standards:

Stock solutions of 1 mg/ml of reserpine and methyl-18-triethylbenzoylreserpate (TEBR) were prepared in methanol and stored refrigerated. Working standards were prepared in 0.01 N HCl in concentrations of 1 ng/ul and 0.1 ng/ul. Stock solutions of 1 mg/ml of reserpic acid, syringomethylreserpate and methylreserpate were prepared in methanol and further diluted to 1 ng/ul and 0.1 ng/ul in 0.01 N HCl.

Instrumentation:

Chromatography was performed with a Model 6000A solvent delivery system, and a Model U6K manual injector or WISP 710A automatic injector (Waters Associates, Milford MA). The column was 4.6 mm i.d. x 25 cm packed with 5 u particle size trimethylsilyl material (LC-1, Supelco, Belafonte, PA). A Model 204 Fluoresence spectrophotometer with an accessary flow cell (Perkin-

Elmer, Norwalk, CT) was used to detect the fluorophors and the response was recorded on an Omniscribe strip-chart recorder at a 10 mv input (Houston Instruments, Austin, TX).

## METHODS

Sample Extraction:

To three (3.0 mls) of plasma, 45 ul (4.5 ng) of internal standard (methyl-18-triethoxybenzoyl reserpate), 1.0 ml carbonate buffer (0.6 M, pH 9.5) and 10 mls of 1.5% (v/v) isoamyl alcohol in n-heptane were added. The mixture was shaken for 10 minutes and centrifuged for 10 minutes. The organic layer was then transferred to 15 ml-tapered centrifuge tube containing 1.2 ml 0.1 N HCl. After shaking and centrifuging for 10 minutes, the top layer was aspirated and the aqueous layer transferred to a 3 ml tapered glass stoppered minicentrifuge tube (fine bore capillary). The contents were made alkaline with 0.5 ml carbonate buffer (0.6 M, pH 9.5) and extracted with 0.5 ml of methyl-tert-butyl ether. After mixing and centrifuging for 5 minutes, the lower layer was discarded and the ether layer transferred to small 1.0 ml vials or to "low volume inserts" if automatic sampling was desired. After evaporation to dryness in a vacuum concentrator (Model SVC-100 M Speed-Vac concentrator, Savant Instruments, Hicksville, NY), the residue was redissolved and reacted at ambient temperature for a minimum of 10 minutes with 40 ul of a 1 in 10 mixture

of a saturated solution of vanadium pentoxide in methanol. The contents were then injected on column.

Chromatographic conditions:

The mobile phase consisted of 50% acetate buffer (0.1 M, pH 4.2) and 50% acetonitrile with addition of 5 mM heptane sulfonate and 0.01 M triethylamine. The flow rate was 1.6 mL/min and temperature ambient. The effluent was monitored via the Model 204 spectrofluorimeter, and excitation and emission wavelengths were 390 nM and 480 nM, respectively.

Quantitation:

The peak height ratios of reserpine to the internal standard were plotted against concentration. A least square linear regression analysis of these data were used to calculate the parameters slope, x-intercept, correlation coefficient, and standard error.



## RESULTS AND DISCUSSION

Reserpine was extracted from 3 ml of plasma, oxidized to a highly fluorescent derivative and chromatographed using a reversed phase ion paired system. The use of the internal standard (methyl-18-triethoxybenzoyl reserpate) which undergoes extraction and derivatization similar to that of reserpine, is ideal since it elutes sufficiently far away from reserpine as well as its major metabolites. The chromatography is rapid enough to permit a large number of samples to be analyzed in a single working day

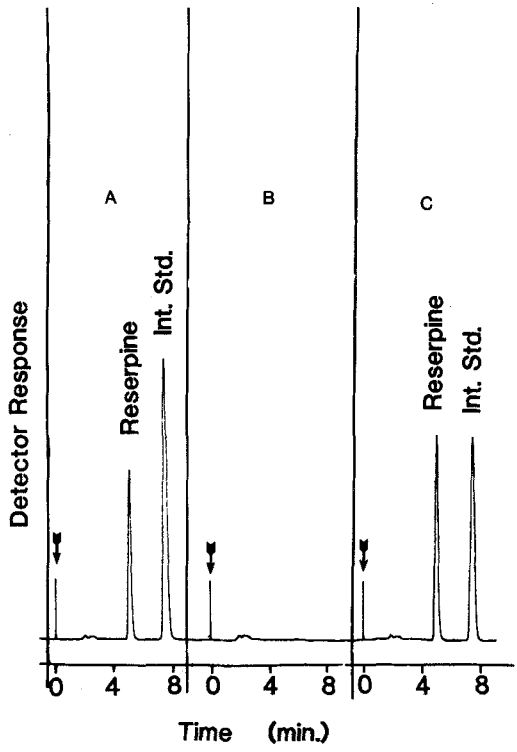


Figure 1A Sample chromatogram of a 3 ml spiked plasma extract containing 1.0 ng/ml reserpine. Entire (40 ul) reconstituted extract was injected. Chromatographic conditions as described in text.

Figure 1B Sample chromatogram of a blank 3 ml plasma extract. Entire reconstituted extract was injected.

Figure 1C Sample chromatogram of a 3 ml patient plasma extract containing 1.62 ng/ml 1 hour after a 0.5 mg i.m. dose of reserpine.

(Fig. 1a). Due to an efficient sample clean-up and the added specificity of fluorescence detection all plasma samples produced a clean chromatogram with no interferring endogenous peaks and a minimal solvent front (Fig. 1b). Actual treated patient plasma

samples did not indicate any interferring peaks. A typical chromatogram appears in Fig. 1c.

Although the lowest concentration for the calibration curve is 0.3 ng/ml, levels below this (ca 0.1 ng/ml) could be detected semi-quantitatively because of the low intercept and high signal-to-noise ratio of this detector.

The absolute recovery of reserpine was carried out by spiking 3 ml of plasma with various concentrations of reserpine. The extraction was carried out quantitatively. The internal standard was added after evaporation and taken to dryness. The residue was derivatized and the extract injected on column. The same standards (and internal standard) were dried down, derivatized and injected in the same concentrations. The difference between the standard and the internal standard in the processed samples compared to the direct-injection sample gave a measure of the overall recovery (Table 1).



The precision of the reported procedure was determined by spiking seven 3 ml aliquots of drug-free plasma with various con-

Table 1

Recovery of Reserpine from 3 ml of Plasma (n=7)

| Concentration (ng/ml) | Percent Recovery | C.V.% |
|---|---|---|
| 0.3 | 82.7 | 7.1 |
| 1.0 | 79.8 | 6.5 |
| 6.0 | 78.4 | 5.5 |

centrations of reserpine. The samples were processed and chromatographed as described. The results appear in Table 2.

A measure of the stability of the assay is demonstrated by the variability in the slope of the linear regression curves on 6 different days (Table 3).

The principle metabolites of reserpine have been reported as reserpic acid, syringomethyl reserpate, and methyl reserpate (1).These structurally related metabolites were tested to determine if any interference occurred. All three reacted with the vanadium pentoxide derivatizing reagent resulting in fluorophors detectable at the same wavelength as reserpine. However, when injected directly these compounds were separated under normal chromatographic conditions (Fig. 2a).Mobile phase



Table 2

Within-run Precision of Assay (n=7)

| Concentration ng/ml | C.V.% |
|---|---|
| 0.3 | 8.5 |
| 1.0 | 8.9 |
| 3.0 | 4.2 |

Table 3

Day-to-Day Stability of Assay Based Upon Slope of the Linear Regression Curves (n=6).

| Slope | + S.D. | C.V.% |
|---|---|---|
| 1.09 | 0.098 | 8.9 |

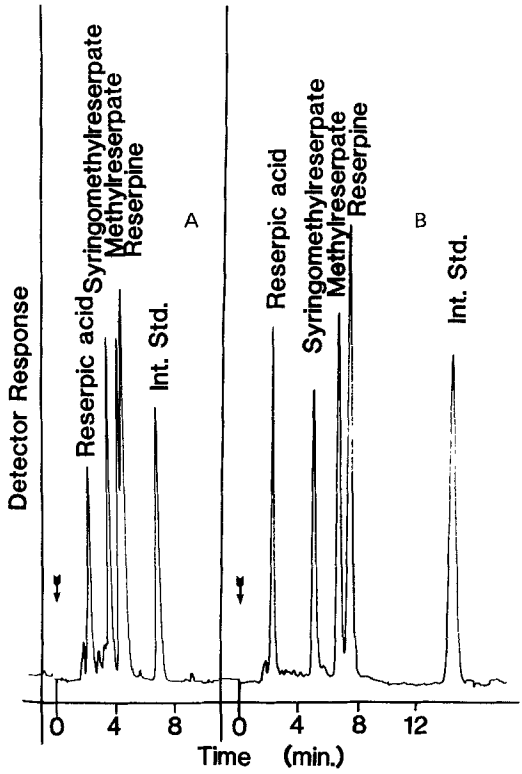


Figure 2A A chromatogram showing 10 ng each of reserpine and the three major metabolites after derivitization with vanadium pentoxide. Chromatographic conditions as described in text.

Figure 2B A chromatogram of the same compounds separated by a change in the mobile phase from 50-50 to 60-40 Acetate-Acetonitrile mixture, all other conditions remained the same.

modification permitted nearly baseline resolution of all compounds (Fig. 2b). Furthermore the only metabolite which was extracted at this pH by this procedure was methyl reserpate. This metabolite was not found in the plasma of > 20 different

patients receiving 0.5 mg i.m. of reserpine with samples taken from 15 minutes to 24 hours post dose. Recovery of the acidic metabolites reserpic acid and syringomethyl reserpate as well as the other fluorophors was achieved by a single extraction with methyl-t-butyl ether from plasma buffered at pH 4.0 .

Two subjects were injected with 0.5 mg i.m. of reserpine and followed for 24 hours post-dose (Fig. 3). Peak plasma concentrations occurred almost immediately (15 minutes) and were still detected at the final time point (24 hours). These data are consistent with previously reported results (2).



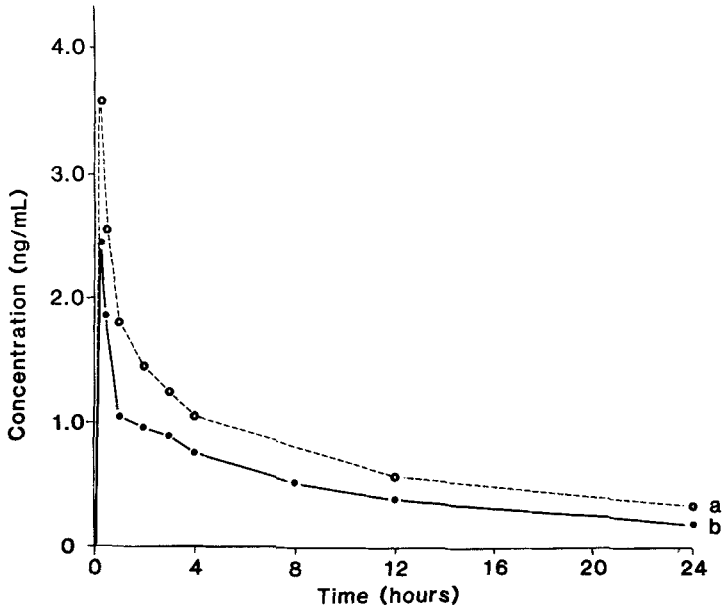


Figure 3 Plasma concentration - time curve of reserpine in two different subjects a, b, each receiving 0.5 mg i.m. reserpine.

In summary, a specific, rapid and sensitive method has been developed which incorporates the merits of two previous procedures. This resulted in a shortened analysis time without sacrificing overall precision or sensitivity.

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