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Design of a photoionization detector for high-performance liquid chromatography using an automated liquid-to-vapor phase interface and application to phenobarbital in an animal feed and to amantadine

JOHN T. SCHMERMUND
Superpharm Corporation, 1769 Fifth Avenue, Bayshore, NY 11706 (U.S.A.)
and
DAVID C. LOCKE*
Chemistry Department, Queens College and The Graduate School, The City University of New York, Flushing, NY 11367 (U.S.A.)
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SUMMARY

An automated liquid-to-vapor phase interface system forms the basis for a new high-performance liquid chromatography (HPLC)–photoionization detection (PID) system. The system incorporates a six-valve interface enabling peak trapping, solvent switching and thermal desorption of the solute of interest into a vapor phase PID. For reversed-phase HPLC, the eluted solute peak is isolated on a Tenax trap after dilution of the effluent with water; the water is then evaporated, following which the trapped solute is flash-evaporated into the PID system. For normal-phase HPLC, the column effluent is diluted with hexane, the solute peak is concentrated on a short column packed with a propyl-amino/cyano bonded phase and the solvent is evaporated. The solute is then eluted with water onto the Tenax trap, and the above procedure for reversed-phase HPLC followed. All operations are controlled with a microcomputer. The advantages of the new detector system include completely automated operation, fast sample preparation, high sensitivity, and inherent selectivity. The system was applied to phenobarbital, which was extracted with acetonitrile from spiked laboratory animal feed, and to amantadine. The phenobarbital assay used a normal-phase separation with hexane–methyl *tert.*-butyl ether–methanol eluent. The manual sample preparation time was 5 min and the limit of detection was 2 ng phenobarbital injected; a conventional HPLC assay with UV detection required a longer sample preparation time and had a detection limit of 700 ng. Amantadine was assayed using a reversed-phase HPLC system with a water–methanol–triethylamine–orthophosphoric acid mobile phase. The detection limit was 25 ng injected.

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INTRODUCTION

In quest of the sensitive, universal detector for high-performance liquid chromatography (HPLC), we have earlier evaluated two modes of detector based on photoionization. The first[1] involved the continuous flash evaporation of the total HPLC effluent into a conventional vapor-phase photoionization detection (PID) system. The device was sensitive to about 10^{-11} g/s of solutes, with a linear dynamic range of 10^5, but suffered the limitation that it was effectively restricted to normal-phase systems. Driscoll *et al.*[2] improved upon these results somewhat by using a commercial PID system and effluent splitting. Our second design[3] attempted direct measurement of liquid-phase photoionization currents. This mode of operation was also restricted to normal-phase HPLC. Other designs of liquid-phase PID systems using high-power, pulsed-laser sources for two-photon ionization processes have also been described[4–10].

We describe here an improved version of the HPLC effluent-evaporation, vapor-phase PID system, which is amenable to both normal- and reversed-phase HPLC. The deleterious effects of the solvent vapors on PID are circumvented by trapping the peak of interest, evaporating off the solvent and then flash-evaporating the solute peak into a gas-phase PID system in a stream of argon. For quantitative solute trapping efficiency, effluent dilution is usually required. With reversed-phase HPLC, the effluent is diluted with water before the solute is trapped on Tenax. Tenax was selected because it has little affinity for water but adsorbs organic compounds and is commonly used to trap organics from water samples[11]. It is also one of the few adsorbents amenable to repetitive, rapid heating for fast solute desorption, without decomposing itself[12]. The water is then evaporated and the solute flashed into the PID system. With normal-phase HPLC, the effluent is first diluted with hexane, the peak trapped on a short column packed with propyl-amino/cyano (PAC) bonded phase and the solvent evaporated in a stream of nitrogen. The sample component is then displaced with water onto a Tenax trap and the operation completed as above. The entire system is microprocessor-controlled for completely automated operation.

Illustrative applications of the system are given to the determination of phenobarbital [5-ethyl-5-phenyl-2,4,6-(1H,3H,5H)-pyrimidine trione] and of amantadine (tricyclo-3.3.1.1.^{3,7}-decan-1-amine), the former spiked into a complex test matrix, laboratory animal feed. The system is more sensitive and more rapid than currently used procedures and allows avoidance of the extensive manual cleanup procedures used in most established HPLC methods for drug substances in animal feeds[13–18].

The system is another example of the application of valve-switching techniques to HPLC. Various applications of a different type of microprocessor-controlled valve-switching unit for automated sample cleanup and trace component enrichment were described by Little *et al.*[19]. Conley and Benjamin[20] devised an automatic column-switching technique using a pneumatically-operated six-port valve for on-line cleanup and analysis of drugs in topical cream formulations. An automated heteromodal column-switching HPLC system using silica and cyano-bonded columns and fluorescence detection was developed by Cox and Pullen[21] for the determination of E-prostaglandin panacyl ester derivatives.

The present system combines sample cleanup and sensitive PID in a unique interface. The limitations of the system include (a) the complexity and expense of the

apparatus, (b) the requirements that the solute be quantitatively trapped by the Tenax and subsequently quantitatively thermally desorbed from it and that the Tenax be stable over many thermal cycles, and (c) the practical ability to handle only a limited number of peaks. However, in many applications in pharmaceuticals, pesticide residues, food, quality control, clinical and process studies, etc., there is only one component of interest in an otherwise complex matrix.

SYSTEM DESIGN

To isolate a chromatographic peak of interest from reversed- and normal-phase HPLC columns and then to detect it with vapor-phase PID requires the ability to

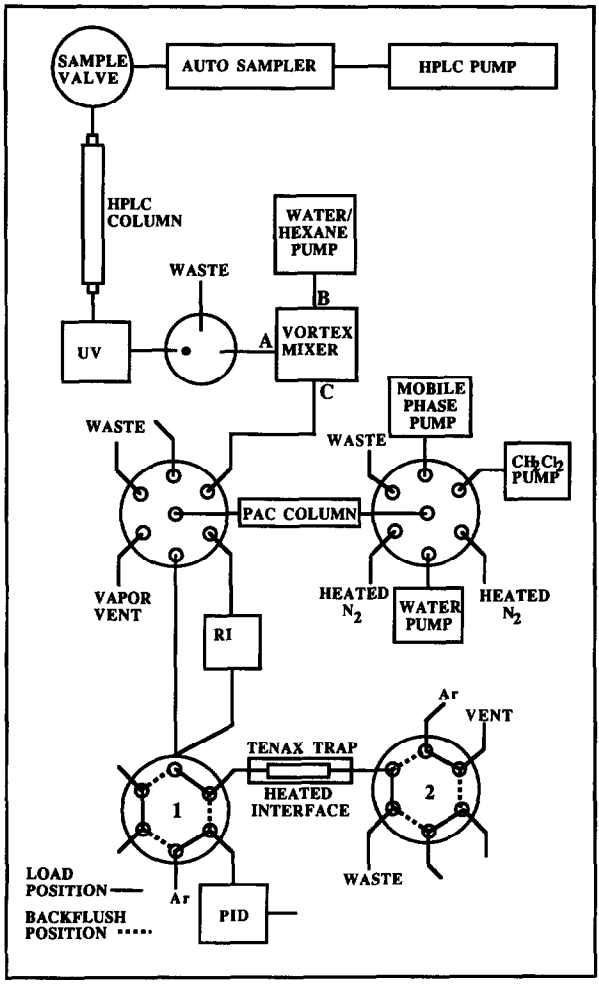

Fig. 1. Diagram of the liquid-to-vapor phase interface for HPLC–PID. Load position refers to trapping of solute peak onto the Tenax trap. Backflush position refers to displacement of solute from Tenax trap to PID system. See text for discussion of the arrangement.

switch columns, change HPLC modes, control liquid flows and thermally desorb the HPLC solvent and the solute in independent steps. We have achieved this capability with a novel interface using up to six valves. The sequencing requires a microprocessor controller, programmed to control the various devices in the system in a fixed timing sequence. A stable and reproducible chromatographic system is therefore required. The hardware is described first, then the controller system and finally applications to two representative systems are given.

HPLC and trapping system

A block diagram of the overall fluid and electrical control systems of the apparatus is shown in Fig. 1. The HPLC pump was a DuPont Model 870. Samples (10 μl) were injected into the HPLC column with a Micromeritics 725 autosampler in which the Micromeritics valve was replaced with a Rheodyne Model 7000 valve. The former valve tended to clog and was not reproducible. The HPLC column was thermostatted in a DuPont column oven. For normal-phase HPLC, a 25 cm × 4.6 mm I.D. column packed with 10-μm Whatman Co:Pell PAC was used at 25°C; for RP-HPLC, the column was a 25 cm × 4 mm I.D. DuPont Zorbax C_8 column at 25°C. The variable-wavelength UV detector used to optimize the chromatographic separation of the phenobarbital was a Kratos Model 773. To facilitate solute peak trapping, the HPLC column effluent was mixed with the diluent, a weak solvent (hexane in the case of normal-phase HPLC; water in reversed-phase HPLC), using an Altex vortex dynamic mixer. The mixer was modified to convert the original cylindrical mixing compartment into a conical shape which provided more efficient mixing. Although the mixer's dead volume was *ca.* 100 μl, peak broadening considerations are generally not as important here as in other HPLC applications, because the peak is trapped prior to detection. Diluent was pumped into port B using a Beckman Model 110A metering pump under the control of the microprocessor and was blended with column effluent entering port A to cause vortexing motion in the conical section of the mixer. The concentration of the stronger solvent in the eluent [methyl *tert.*-butyl ether (MTBE)–methanol in normal-phase HPLC; acetonitrile in reversed-phase HPLC] could be reduced up to 75% using this system.

The trapping or concentrator column which isolated the peak of interest from the effluent was a 3 cm × 4.6 mm I.D. column packed with 30–38-μm Whatman Co:Pell PAC. This trap was used only in the normal-phase HPLC mode. Depending on the chemical nature of the peak of interest, alternative adsorbents could be used. The concentrator column was connected as shown in Fig. 1 to the two tandem, pneumatically operated, actuator-controlled Rheodyne Model 5704 valves. Compressed air at 28–30 p.s.i. drove the valves on command from the microprocessor. This part of the sequence started with the hexane-diluted normal-phase HPLC effluent from the vortex mixer being pumped through the concentrator column to trap out the peak (flow was left-to-right through port 1). The valve was then advanced to allow hexane solvent to be evaporated by a back-flow of warm nitrogen to dry the packing material (flow was now right-to-left, to vent). In the third valve position, the trapped solute was backflushed from the dried PAC concentrator colum to the Tenax trap with water pumped from a second Beckman 110A metering pump. After quantitative transfer, the PAC column was regenerated by successive valve advances. First, heated nitrogen through port 3 drove most of the water from the concentrator col-

umn; dichloromethane was then pumped through port 4 using a third Beckman 110A pump to displace the remaining water. Finally the PAC concentrator column was equilibrated with 6–7 column volumes of the normal-phase eluent in preparation for the next fraction switched from the analytical HPLC column. The Hewlett-Packard refractive index (RI) detector indicated on Fig. 1 was used to determine the time required to purge solvent from the colum with nitrogen and also to optimize the HPLC separation of the UV-transparent amantadine solute.

The Tenax trap now contained the solute band eluted from the PAC concentrator column and was connected as shown in Fig. 1 for load and backflush positions. These valves were Carle Instruments Model 5621 valves housed in a Carle Model 4300 valve oven. The valves and transfer lines had to be maintained at a temperature at least as high as the desorption temperature to prevent condensation of solute vapors. The valves were held in the load position until most of the water was flushed from the valve to waste in the nitrogen stream and were then rotated under control of the microprocessor to the backflush position. The trap was 3 cm × 4.6 mm I.D., dry-packed with 80–100 mesh Tenax-GC (Alltech Assoc.) which had been conditioned in a vacuum oven at 250°C for 24 h to remove any volatiles. The Carle valve oven was mounted onto the front of a Perkin-Elmer Model 3290 gas chromatography (GC) system. The GC system served as a convenient device to connect the trap to the H Nu Model PI-52-02 PID system via a 3 cm × 4.6 mm I.D. glass-lined stainless-steel tube. The PID system was mounted atop the GC system adjacent to the valve oven. The PID discharge tube contained Kr, which produced an intense line of 10.2 eV energy. Signals from the H Nu PID electronics were recorded on a Hewlett-Packard 3390 integrator.

Thermal desorption unit

The Tenax trap was contained in the thermal desorption unit which was constructed of an aluminum block into which holes were drilled for a cartridge heater, a linear thermistor (linistor) and the Tenax trap. The unit was controlled by a contact closure from the microprocessor output board. The cartridge heater was powered with a precise temperature controller using a closed-loop proportional feedback system incorporating the linistor, based on an RCA circuit[22]. The temperature accuracy of this controller was of the order of ± 0.2°C and depended on the input sensitivity of the RCA CA 3059 hybrid integrated circuit, the specific linistor used and the level of temperature being controlled. A range of temperatures up to 300°C could be provided. The microprocessor turned the circuit on and rapidly ramped the temperature up to the preset value. The microprocessor quartz crystal clock monitored the duration of the preset and desorption temperatures before the cool-down process at the programmed time.

Microprocessor controller

The system was controlled using an expanded Systec SLIC-1400 microcomputer. The 8-bit central processing unit was based on a 6502 chip, which regulated all operations of the microcomputer based on the sequence of instructions programmed into memory from a keyboard. Timing by the unit's quartz crystal clock synchronized data transfer. The program was started to run by a contact closure on the Micromeritics autosampler. Both the analog and digital outputs of the SLIC-1400 were utilized.

The analog output provided a continuously variable voltage which was used to control the pumps in Fig. 1. For example, a controlled-voltage ramp allowed the HPLC column pump to reach a specified flow-rate over a designated time interval to avoid a rapid pressure surge that could damage the column. The digital output was cascaded to provide sixteen individual channels. Each channel contained a 10-A relay which provided a contact closure to switch a valve or to start a thermal cycle. Time intervals for the contact closures were programmed from the keyboard.

The system described is amenable to considerable simplification. A personal computer could substitute for the SLIC-1400. An oven simpler than a GC system would suffice to connect the Tenax trap to the PID system. The refractive index detector was a one-time requirement for a UV-transparent solute; similarly, a UV detector need not be dedicated to this system but is used only to optimize chromatographic conditions. If only reversed-phase HPLC is to be done, which is the usual case in pharmaceutical analyses, provision for solvent-switching is obviated, which eliminates the need for two of the Beckman pumps and one valve.

Materials

Phenobarbital was United States Pharmacopoeia (U.S.P.) reference material. Solutions were prepared every 12 h, because solutions of the compound are unstable. Amantadine was also U.S.P. reference grade. All solvents were Burdick & Jackson distilled-in-glass HPLC grade. Animal feed samples were Purina Laboratory Chow (Ralston-Purina, St. Louis, MO, U.S.A.). Spiking was carried out by adding 5.0 ml of a 1-mg/ml solution in acetonitrile–water (1:1) to 10.0 g of ground feed, which was then agitated mechanically in a flask and the solvent evaporated at ambient temperature in a Büchi Rotovap. The internal standard used was 4-hydroxy benzoic acid heptyl ester (heptyl paraben).

RESULTS AND DISCUSSION

Phenobarbital assay

HPLC. Phenobarbital can be determined using either reversed-phase HPLC or normal-phase HPLC. Reversed-phase HPLC was used to study the animal feed extraction solvent. However, the automated PID study was conducted using normal-phase HPLC, because (a) the polar materials coextracted with the phenobarbital were more easily separated using normal-phase HPLC and (b) normal-phase HPLC represented a more difficult challenge for the new PID system. The reversed-phase HPLC eluent composition was established by systematically varying the proportions of water, glacial acetic acid and acetonitrile. The best separation of the phenobarbital and the heptyl paraben internal standard with good peak symmetry was obtained with water–acetonitrile–acetic acid (69:30:1, v/v/v) at 2.0 ml/min. For normal-phase HPLC, a series of solvent optimization experiments led to the choice of a normal-phase eluent containing hexane–MTBE–methanol (79:18:3, v/v/v).

Extraction study. Animal feed samples (10.0 g) spiked with 5.0 mg of phenobarbital were extracted 3 times for 1 h with 100 ml of the solvents below. Extracts were decanted through silanized glass wool. The extract solutions were evaporated to dryness at 35°C using a Büchi Rotovap. To each were then added 5.0 ml of a 0.1-mg/ml solution of heptyl paraben internal standard in acetonitrile–water (1:1) and the resi-

due dissolved with mechanical shaking for 10 min. Solutions were placed into HPLC autosampler vials and analyzed using the reversed-phase HPLC system with a UV detector at 235 nm. Ratios of the areas of phenobarbital to heptyl paraben peaks were compared with a linear calibration plot covering the range of 0.10–10 μg phenobarbital and 0.01–1.0 μg heptyl paraben in the 10μl aliquot injected. The solutions were prepared using dilutions of a solution containing 1 mg phenobarbital plus 0.1 mg heptyl paraben per ml of acetonitrile–water (1:1, v/v). Of the extraction solvents tested, *i.e.* hexane, MTBE, dichloromethane, chloroform, ethyl acetate, acetonitrile, acetone and methanol, acetonitrile gave reasonable recovery [71.9% ± 1.5% relative standard deviation (R.S.D.) ($n=3$)] with minimal extraction of extraneous feed matrix components. Acetone and methanol gave higher recoveries (90%) but extracted lipophilic materials that interfered with the chromatographic determination of the phenobarbital. The other solvents gave poorer recoveries than acetonitrile. The purity of the phenobarbital peak was confirmed by collecting the peaks after repetitive injections, evaporating the solvent and using direct-inlet probe mass spectrometry. The mass spectrum of the combined collected peaks was identical to that of a sample of pure phenobarbital.

Normal-phase HPLC concentrator column. Seven different commercially available HPLC packing materials were tested for use as concentrator column packings. Each was dry-packed into short (3 cm × 4.6 mm I.D.) stainless-steel tubes. Each concentrator column was connected in turn to the effluent end of the vortex mixer, which was itself connected to the normal-phase HPLC column. A 10-μl sample of phenobarbital spiking standard was injected into the HPLC column and eluted with 2.0 ml/min of the normal-phase mobile phase. The polarity of the effluent was reduced by dilution with hexane to facilitate trapping of solute on the concentrator packing; in the vortex mixer, 1.0 ml/min of hexane was pumped into the HPLC effluent stream. The effluent from the concentrator column was monitored with the Kratos UV detector at 235 nm. The retention times of phenobarbital on the concentrator columns tested were: Baker diol, 2.3 min, DuPont Permaphase ETH, 3.5 min, Baker cyano, 5.3 min, Whatman Pre-Col silica gel, 5.7 min, DuPont Zorbax BP-cyano, 6.2 min, Whatman HC Pellosil silica gel, 7.0 min and Whatman Co:Pell PAC, > 30 min. Thus, as noted above, the Whatman Co:Pell PAC material was selected because phenobarbital has a retention volume greater than 90 ml, more than adequate for our purposes.

Displacement of phenobarbital onto the Tenax trap. A 10-μg phenobarbital peak was isolated on the PAC concentrator column from the HPLC-resolved feed extract as described above. First, the column was dried for 10 min in a stream of warm nitrogen. To determine the volume of water required to elute the phenobarbital from the concentrator column, the UV detector was connected to the column and water was pumped in at 1.0 ml/min. Phenobarbital eluted as a narrow peak in 10 min.When the Tenax trap was inserted between the UV detector and the PAC concentrator column, phenobarbital did not elute from the latter after 20 min, *i.e.* it was quantitatively trapped on the Tenax column. In practice, water was pumped at 1.0 ml/min for 12.0 min to effect transfer from PAC to Tenax traps.

Thermal desorption from Tenax into the PID system. Samples of phenobarbital (0.5 μg) were transferred from the HPLC column to the Tenax trap installed in the Perkin-Elmer 3290 GC oven between the carrier gas inlet and the PID system accord-

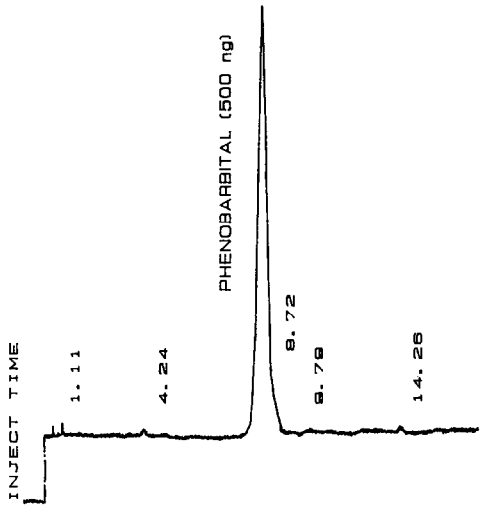

Fig. 2. Optimized desorption of phenobarbital from Tenax. Conditions: Perkin-Elmer 3290 GC oven, temperature raised from 50 to 150°C in 3 min; carrier gas, Ar, 55 ml/min; PID. Values given are retention times in min.

ing to the above operation and the water removed in a stream of warm nitrogen. The temperature was stabilized at 50°C and then programmed to 150°C over 3 min. The PID system detected a single peak corresponding to phenobarbital, as illustrated in Fig. 2.

Comparison with a conventional HPLC method. A published study of the determination of phenobarbital in animal feed[14] used a methanol extraction followed by sequential liquid–liquid extractions at pH 13 and pH 1, a silica gel column cleanup and reversed-phase HPLC (water–methanol, 60:40) with UV detection at 210 nm. We found this method required analyst preparation time of about 15 min/sample, and, on a routine basis, gave a lower limit of detection of 0.14 ppm and an assay precision of 1.4% (R.S.D., $n = 3$).

The automated HPLC–PID method described here required only about 5 min of analyst sample preparation time per sample. The detection limit based on the injected weight of phenobarbital required to produce a peak twice the noise level was 2 ng. This would correspond to a concentration in the feed of 0.06 ppm. However, we should note the lowest level of spiking for which the entire method was tested was 25 ppm; whether extraction recovery is satisfactory at much lower levels will have to be demonstrated. The present work was carried out to evaluate the new detection system and the feasibility of applying it to a complex matrix, rather than to validate a new specific analytical method. The overall precision of the automated HPLC–PID procedure was 2.4% (R.S.D., $n = 3$) at the spiking level of 500 μg/g of animal feed.

Amantadine assay

A brief study of the behavior of amantadine in this system was also conducted to illustrate the application of the automated HPLC–PID method in the reversed-phase mode. The conventional assay for amantadine used packed-column GC with a

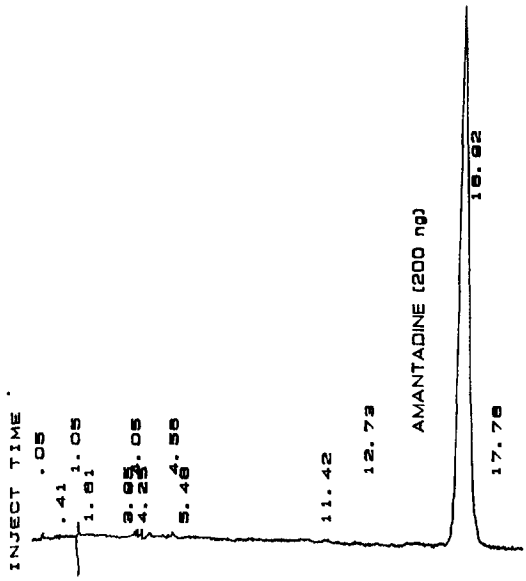

Fig. 3. Optimized desorption of amantadine from Tenax. Conditions: same as Fig. 2 except temperature varied from 50 to 180°C in 4 min and Ar flow at 40 ml/min.

flame ionization detector[23]. Kirschbaum[23] also described briefly an HPLC method requiring derivatization of the amantadine to phthalimido-adamantane followed by reversed-phase HPLC on a C_{18} column with methanol–water–85% orthophosphoric acid (60:40:0.1, v/v/v) and a UV detector operated at 254 nm. Amantadine itself is transparent in the UV.

Reversed-phase HPLC. The mobile phase of Kirschbaum[23] was modified by the addition of 1% (v/v) of triethyl amine. The triethylamine improved symmetry of the underivatized amantadine peak by acting as a competing base and seemed to ameliorate the other problems noted by Kirschbaum[23] in the HPLC of the free base. To set up the automated PID method, the refractive index detector was used.

Trapping on Tenax. With reversed-phase mobile phases, the column effluent need only be diluted with water prior to trapping on the Tenax, thus eliminating the need for a concentrator column and phase-switching. The effluent and water streams were combined in the vortex mixer before passing into the trap. It was found that a 100-μg sample of amantadine was retained in the Tenax trap more than 15 min when 2.0 ml/min of eluent diluted with 1.5 ml/min of water was passed through it.

Thermal desorption of amantadine-PID Amantadine is thermally stable and eluted from the Tenax in the heated interface at 180°C in argon at 40 ml/min into the PID system. A typical chromatogram is shown in Fig. 3.

Sensitivity and precision. The precision of the new assay procedure for amantadine determined by injection into the HPLC of 10-μl aliquots of a 20-μg/ml solution of amantadine in methanol was 1.5% (R.S.D., $n = 3$). Based on the quantity of amantadine required to give a signal twice the noise level, the limit of detection using the automated HPLC–PID method was 25 ng amantadine injected. The limit of

detection of an overall method including extraction from spiked animal feed has yet to be determined.

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